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(54) Title: TREATMENT OF CANCER USING CYTOKINE–EXPRESSING POLYNUCLEOTIDES AND COMPOSITIONS THEREFOR

(57) Abstract

The present invention provides a pharmaceutical composition, comprising a non–infectious, non–integrating polynucleotide construct comprising a polynucleotide encoding an interferon ω and one or more cationic compounds. The present invention also provides methods of treating cancer in a mammal, comprising administering into a tissue of the mammal a non–infectious, non–integrating polynucleotide construct comprising a polynucleotide encoding a cytokine. In addition, the present invention also relates to the methodology for selective transfection of malignant cells with polynucleotides expressing therapeutic or prophylactic molecules in intracavity tumor bearing mammals. More specifically, the present invention provides a methodology for the suppression of an intra–cavity dissemination of malignant cells, such as intraperitoneal dissemination.

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Treatment of Cancer Using Cytokine-Expressing Polynucleotides and Compositions Therefor

Background of the Invention

Field of the Invention

The present invention relates to treatment of cancer in mammals. Generally, the present invention provides methods of treating cancer in a mammal by administering a polynucleotide construct comprising a polynucleotide encoding a cytokine. In addition, the present invention relates to the methodology for selective transfection of malignant cells with polynucleotides expressing therapeutic or prophylactic molecules in intra-cavity tumor bearing mammals. More specifically, the present invention provides a methodology for the suppression of an intra-cavity dissemination of malignant cells, such as intraperitoneal dissemination.

Related Art

Cytokines have been demonstrated both in pre-clinical animal models as well as in humans to have potent anti-tumor effects. In particular IFN's have been tried for the treatment of a number of human concerns.

The interferons (IFNs) are a family of cytokines with potent anti-viral, antiproliferative, and immunomodulatory activities and play important roles in the body's defensive response to viruses, bacteria, and tumors (Baron, S. *et al.*, *JAMA 266*:1375 (1991)). On the basis of antigenicity, biochemical properties, and producer cell, the interferon's have been divided into two classes, type I interferon and type II interferon. IFNα, IFNβ, IFNω, and IFNτ are type I interferons, and bind to the same α/β receptor. IFNγ is a type II interferon, and binds to the γ receptor (Pestka, S., *Ann. Rev. Biochem. 56*:727 (1987)). IFNα and IFNβ are naturally expressed in many cells upon viral infection. IFNγ is produced by activated T lymphocytes and natural killer (NK) cells.

IFNτ is believed to possess hormone activity, and plays an important role in pregnancy in cattle, sheep, and related ruminants (Imakawa, K. *et al.*, *Nature 330*:377 (1987); Stewart, H.J. *et al.*, *J. Endocrinology 115*:R13 (1987)). Due to the pleiotropic activities of IFNs, these cytokines have been studied for their therapeutic efficacy in a number of diseases, particularly cancers and viral infectious diseases.

IFNω was discovered independently by three different groups in 1985 (Capon, D.J., *et al.*, *Molec. Cell. Biol. 5*: 768-779 (1985), Feinstein, S. *et al.*, *Molec. Cell. Biol 5*:510 (1985); and Hauptmann and Swetly, *Nucl. Acids Res. 13*: 4739-4749 (1985)). Unlike IFNα, for which at least 14 different functional nonallelic genes have been identified in man, IFNω is encoded by a single functional gene. IFNω genes are believed to be present in most mammals, but have not been found in dogs, rats or mice. The mature IFNω polypeptide is 172 amino acids and shares 60% sequence homology with the human IFNα's. Due to the sequence similarity with IFNα, IFNω was originally considered to be a member or a subfamily of IFNα, and was originally termed IFNα-$_{II}$. IFNω is a significant component (≈10%) of human leukocyte-derived interferon, the natural mixture of interferon produced after viral infection (Adolf, G. *et al.*, *Virology 175*:410 (1990)). IFNω has been demonstrated to bind to the same α/β receptor as IFNα (Flores, I. *et al.*, *J. Biol. Chem. 266*: 19875-19877 (1991)), and to share similar biological activities with IFNα, including anti-proliferative activity against tumor cells *in vitro* (Kubes, M. *et al.*, *J. Interferon Research 14*:57 (1994) and immunomodulatory activity (Nieroda *et al.*, *Molec. Cell. Differentiation 4*: 335-351 (1996)).

Recombinant IFNα polypeptide has been approved for use in humans for hairy cell leukemia, AIDS-related Kaposi's sarcoma, malignant melanoma, chronic hepatitis B and C, chronic myleogenous leukemia, and condylomata acuminata (Baron, S. *et al.*, *JAMA 266*:1375 (1991)). However, for each of these indications, IFNα polypeptide must be administered repeatedly, often on a daily basis, for extended periods of time to maintain effective serum levels

due to the short half-life (hours) of the polypeptide in the serum (Friedman, *Interferons: A Primer*, Academic Press, New York, pp. 104-107 (1981); Galvani and Cawley, *Cytokine Therapy*, Cambridge University Press, Cambridge, pp. 114-115 (1992)). Thus, in spite of producing clinical benefit for many disease conditions, the use of IFNα polypeptide is associated with acute and chronic side effects in most patients (Jones, *Cancer 57*: 1709-1715 (1986); and Quesda *et al.*, *Blood 68*: 493-497 (1986)). The severity of the adverse reaction correlates with peak serum interferon levels.

Viral or plasmid vectors containing IFNα genes have been used in *ex vivo* therapy to treat mouse tumors. For example, tumor cells were transfected *in vitro* with viral or plasmid vectors containing an IFNα gene, and the transfected tumor cells were injected into mice (Belldegrun, A., *et al.*, *J. Natl. Cancer Inst. 85*: 207-216 (1993); Ferrantini, M. *et al.*, *Cancer Research 53*: 1107-1112 (1993); Ferrantini, M. *et al.*, *J. Immunology* 153: 4604-4615 (1994); Kaido, T. *et al.*, *Int. J. Cancer 60*: 221-229 (1995); Ogura, H. *et al.*, *Cancer Research 50*: 5102-5106 (1990); Santodonato, L., *et al.*, *Human Gene Therapy 7*:1-10 (1996); Santodonato, L., *et al.*, *Gene Therapy 4*:1246-1255 (1997)). In another *ex vivo* study, cervical carcinoma and leukemia cells were transfected with a viral vector containing the interferon-consensus gene, and the transfected cells were injected into mice (Zhang, J.-F. *et al.*, *Cancer Gene Therapy 3*: 31-38 (1996)). In all of these *ex vivo* studies, varying levels of anti-tumor efficacy, such as tumor regression and/or prolonged survival, have been observed.

Viral or plasmid vectors containing interferon genes have also been used in *in vivo* therapy for tumor-bearing mice. For example, a viral vector containing the interferon-consensus gene was injected into mice bearing transplanted MDA-MB-435 breast cancer, hamster melanoma, or K562 leukemia, and tumor regression was reported (Zhang, J.-F. *et al.*, *Proc. Natl. Acad. Sci. USA 93*: 4513-4518 (1996)). In a similar study, a plasmid vector containing human IFNβ gene complexed with cationic lipid was injected intracranially into mice bearing a human glioma, and tumor regression was reported (Yagi, K. *et al.*, *Biochemistry and Molecular Biology International*

32: 167-171 (1994)). In a murine model of renal cell carcinoma the direct intratumoral injection of an IL-2 plasmid DNA : lipid complex has been shown to result in complete tumor regression and a significant induction of a tumor specific CTL response increase in survival (Saffran *et al., Cancer Gene Therapy* 5: 321-330 (1998)).

Plasmid vectors containing cytokine gens have also been reported to result in systemic levels of the encoded cytokine and in some cases, biological effects characteristic of each cytokine in mice. For example, the intramuscular injection of plasmid DNA encoding either TGFβ, IL-2, IL-4, IL-5, or IFNα resulted in physiologically significant amounts in the systemic circulation of the corresponding cytokine polypeptide (Raz, E. *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 4523-4527 (1993); Raz, E. *et al.*, *Lupus* 4: 266-292 (1995); Tokui, M. *et al., Biochem. Biophys. Res. Comm.* 233: 527-531 (1997); Lawson, C. *et al., J. Interferon Cytokine Res.* 17: 255-261 (1997); Yeow, W.-S. *et al., J. Immunol.* 160: 2932-2939 (1998)).

U.S. Patent No. 5,676,954 reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355; 4,946,787; 5,049,386; 5,459,127; 5,589,466; 5,693,622; 5,580,859; 5,703,055; and International Patent Application No. PCT/US94/06069 (publication no. WO 04/9469) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international patent application no. PCT/US94/06069 (publication no. WO 04/9469) provide methods for delivering DNA-cationic lipid complexes to mammals.

Even though some viral vectors used in *ex vivo* and *in vivo* cancer therapy in murine models showed anti-tumor efficacy, the use of viral vectors to deliver interferon-expressing genes in vivo could induce anti-viral immune responses or result in viral integration into host chromosomes, causing disruption of essential host genes or activation of oncogenes (Ross *et al., Human Gene Therapy 7*: 1781-1790 (1996)).

For treatment of multiple metastatic carcinomas of a body cavity are treated using laparoscopy (Childers *et al, Gynecol. Oncol.* 59: 25-33, (1995)),

catheterization (Naumann *et al*, *Gynecol. Oncol.* 50: 291-3, (1993)) or other access devices (Almadrones *et al*, *Semin. Oncol. Nurs.* 11: 194-202, (1995)). Treatment is usually by surgical removal of primary and large metastatic tumors and postoperative chemotherapy (Kigwawa *et al*, *Am. J. Clin. Oncol.* 17: 230-3, (1994); Markman *et al*, *J. Clin. Oncol.* 10: 1485-91, (1992)) or radiotherapy (Fjeld et al, *Acta. Obstet. Gynecol. Scand. Suppl.* 155: 105-11, (1992)). Tumor recurrence is monitored by magnetic resonance imaging (Forstner *et al, Radiology* 196: 715-20, (1995)), ascites cytology (Clement, *Am. J. Clin. Pathol.* 103: 673-6, (1995); Forstner *et al*, *Radiology* 196: 715-20, 1995) and blood analyses (Forstner *et al, Radiology* 196: 715-20, (1995)). Many intraperitoneal (i.p.) cancers, such as ovarian cancer, eventually metastasize via the lymphatic system to the lungs or other vital organs, and the prognosis for the patient is very poor (Kataoka *et al*, *Nippon Sanka Fujinka Gakkai Zasshi* 46: 337-44, 1994; Hamilton, *Curr. Probl. Cancer* 16: 1-57, (1992)).

Human ovarian cancer is often diagnosed at an advanced stage when the effectiveness of surgery and chemotherapy are limited. The lack of effective treatment options for late-stage patients warrants the development of new treatment modalities for this disease. There have been several attempts to develop an effective immunotherapy for the treatment of ovarian cancer.

The early work in this area involved mouse studies in which bacteria-derived immunostimulants, such as *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*, were injected i.p. as non-specific activators of the immune system. (Knapp and Berkowitz, *Am. J. Obstet. Gynecol., 128*: 782-786, (1977); Bast *et al., J. Immunol., 123*: 1945-1951, (1979); Vanhaelen, *et al., Cancer Research*, 41: 980-983, (1981); and Berek, *et al., Cancer Research*, 44, 1871-1875, (1984)). These studies generally resulted in a non-specific immune response that often did not prevent the growth of later tumors. In addition, if the bacterial antigens were injected more than 24 hours after tumor cell inoculation, there was minimal antitumor response, suggesting that treatment of late-stage ovarian cancer patients with this type of therapy would not be effective.

More recent studies in both mice and humans have involved the i.p. or intravenous (i.v.) administration of cytokine proteins as more specific activators of the immune response (Adachi, *et al*, *Cancer Immunol. Immunother. 37*: 1-6, (1993); Lissoni, *et al*, *Tumori. 78*: 118-20, (1992)). Treating murine ovarian tumors with a combination of recombinant IL-2 and GM-CSF proteins had some beneficial effect in inhibiting ascites production; however, IL-2 was only effective if it was combined with GM-CSF (Kikuchi, *et al.*, *Cancer Immunol. Immunother.*, *43*: 257-261, (1996)). Similarly, a combination of IL-2 and lymphokine-activated killer (LAK) cells was able to reduce i.p. sarcomas in mice, while IL-2 protein alone was not as effective (Ottow, *et al.*, *Cellular Immunology, 104*: 366-376, (1987)). Human clinical trials evaluating IL-2 protein therapy of ovarian cancer patients indicated some antitumor effects (Chapman *et al.*, *Investigational New Drugs*, 6:179-188, (1988);West *et al.*, *N. Engl. J. Med.* 316:898-905, 1987; Lotze *et al.*, *Arch. Surg.* 121:1373-1379, 1986; Benedetti Panici *et al.*, *Cancer Treatment Review*, 16A:123-127, 1989; Beller *et al.*, *Gynecol. Oncol.*, 34:407-412, 1989; Urba *et al.*, *J. Natl. Cancer Inst.*, 81:602-611, 1989; Stewart *et al.*, *Cancer Res.*, 50:6302-6310, 1990; Steis *et al.*, *J. Clin. Oncol.*, 8:1618-1629, 1990; Lissoni *et al.*, *Tumori*, 78:118-120, 1992; Sparano *et al.*, *J. of Immunotherapy*, 16:216-223, 1994; Freedman *et al.*, *J. of Immunotherapy*, 16:198-210, 1994; Edwards *et al.*, *J. Clin. Oncol.*, 15:3399-3407, 1997).

Recent studies in mice have involved the injection of DNA constructs encoding "suicide" genes followed by treatment with prodrugs. This approach has successfully caused regression of some small tumors but has been less successful on larger tumor masses. (Szala, *et al. Gene Therapy 3*: 1025-1031, 1996; Sugaya, *et al. Hum Gene Ther 6*: 317-323 (1996)). In another study, liposome-mediated *E1A* gene therapy for mice bearing ovarian cancers that overexpress HER-2/*neu* resulted in reduced mortality among these tumor bearing mice. (Yu, *et al. Oncogene, 11*: 1383-1388 (1995)). Similarly, the successful treatment of murine ovarian carcinoma (MOT) has been demonstrated using cisplatin-induced gene transfer of DNA constructs encoding IFNγ via i.p. injection. (Son, *Cancer Gene Therapy 4:* 391-396

(1997)). However, this study demonstrated that tumors were poorly responsive to either the IFNγ gene or cisplatin alone, suggesting that the effectiveness of the cisplatin-based gene therapy protocol was mainly due to enhanced sensitization of cisplatin-exposed tumor cells to transfection by the IFNγ gene. (Son, *Cancer Gene Therapy 4:* 391-396, 1997).

Clearly, there is a need for superior therapeutic compositions and methods for treating mammalian cancer. Further, there is a need for an *in vivo* delivery system for IFNω. The present invention provides a simple and safe yet effective compositions and methods for treatment of mammalian cancer.

The present invention also solves the problems inherent in prior attempts to treat body cavity malignancies. The inventors show herein that the malignant cell dissemination into body cavities, such as into the peritoneal cavity during late stage ovarian cancer, can be suppressed simply by administering as few as two to six doses of a polynucleotide formulation directly into the body cavity. This treatment results in selective transfection of malignant cells, and subsequent long-term local production of an effective amount of therapeutic molecules.

Summary of the Invention

The present invention is broadly directed to treatment of cancer by administering *in vivo*, into a tissue of a mammal suffering from cancer, a polynucleotide construct comprising a polynucleotide encoding a cytokine. The polynucleotide construct is incorporated into the cells of the mammal *in vivo*, and a therapeutically effective amount of a cytokine is produced *in vivo*, and delivered to tumor cells. Combinations of cytokine-encoding polynucleotides can be administered.

The present invention provides a pharmaceutical composition comprising about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group

consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and any of the above group complexed with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The present invention also provides a pharmaceutical composition obtained by complexing a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The present invention also provides a method of treating cancer in a mammal, comprising administering into a tissue of the mammal a non-

infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active fragment thereof, such that the polynucleotide is expressed *in vivo*, and such that the cytokine, or active fragment thereof, is delivered systemically to a tumor tissue in an amount effective to treat the cancer.

The present invention also provides a method of treating cancer in a mammal, comprising administering into a tissue of the mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine selected from the group consisting of interferon-ω, interferon-α, and a combination thereof, such that the polynucleotide or an active fragment thereof is expressed, and such that the cytokine is delivered locally to a tumor tissue in an amount effective to treat the cancer. Preferably, the polynucleotide construct is complexed with a cationic vehicle, more preferably, the cationic vehicle may be a cationic lipid, and most preferably, the cationic lipid may be mixed with a neutral lipid.

Another object of the invention is to provide a method of selectively transfecting malignant cells in a body cavity of a tumor-bearing mammal, comprising administering into the body cavity at least one non-infectious, non-integrating polynucleotide complexed with a cationic vehicle, such that the polynucleotide is expressed substantially in the malignant cells of the body cavity. Preferably, the cationic vehicle comprises one or more cationic lipids, and more preferably, the cationic vehicle comprises a cationic and neutral lipid mixture. In a preferred embodiment, the present invention is used to suppress peritoneal dissemination of malignant cells in a tumor-bearing mammal. In particular, the mammal may have ovarian cancer, or metastasis of ovarian cancer. Preferred polynucleotides may encode cytokines, or active fragments thereof. Most preferably, the polynucleotide may encode IL-2, or an active fragment thereof.

Compared to injection of recombinant cytokine polypeptides, the methods described herein have several important advantages. The present invention shows that *in vivo* transfection of cells with encoding polynucleotide, such as an IL-2 or IFNω, results in serum levels of the

corresponding cytokine that have therapeutic effects, and yet are lower than the maximal serum levels typically required when cytokine polypeptides are injected. Further, injecting frequent high doses of cytokine polypeptides can produce debilitating side effects. The methods of the present invention provide cytokine therapy requiring less frequent injections of cytokine-encoding nucleic acids. The injection of polynucleotide constructs encoding cytokines produces sustained, low levels of biologically active cytokines that have beneficial effects, while minimizing adverse side effects.

Compared to the delivery of cytokine genes via a viral gene delivery vectors, the present method also has important advantages. Injection of non-viral vectors of the present method does not induce significant toxicity or pathological immune responses, as described, for example, in mice, pigs or monkeys (Parker, *et al., Human Gene Therapy 6*: 575-590 (1995); and San, *et al., Human Gene Therapy 4*: 781-788 (1993)). Thus, a non-viral vector is safer and can be repeatedly injected.

Brief Description of the Figures

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures.

FIG. 1 shows the plasmid map of VR4151 (SEQ ID No. 4). The cytomegalovirus immediate-early gene promoter enhancer and 5′ untranslated sequences (5′UTR + intron A) drive the expression of the human interferon ω coding sequence. The transcriptional terminator region includes polyadenylation and termination signals derived from the rabbit β-globin gene.

FIG. 2 shows the pharmacokinetics of hIFNω in the serum of C57BL/6 mice (FIG. 2A) and nude mice (FIG. 2B) after a single intramuscular (i.m.) injection of hIFNω plasmid DNA (VR4151). Mice were injected i.m. with 100 μg of VR4151. Following the intramuscular

injection, mice were bled daily, and serum was collected and assayed for hIFNω polypeptide using an ELISA. Each point represents an average of four mice. In C57BL/6 mice, the single i.m. injection resulted in peak serum levels of 254 pg/ml on day 6 after injection, and serum levels were still detectable 14 days after injection (50 pg/ml) (FIG. 2A). In nude mice, the single i.m. injection resulted in peak serum levels of 648 pg/ml on day 7, and serum levels were still detectable 14 days after injection (134 pg/ml) (FIG. 2B).

FIG. 3 shows that systemic mIFNα treatment reduces tumor volume (FIGs. 3A, 3C, and 3E) and increases survival (FIGs. 3B, 3D, and 3F) in three murine tumor models. C57BL/6 mice bearing subcutaneous B16F10 melanoma (FIGs. 3A and 3B), subcutaneous glioma 261 (FIGs. 3C and 3D), or DBA/2 mice bearing subcutaneous Cloudman melanoma (FIGs. 3E and 3F) were injected with 100 μg either of VR4111 (mIFNα plasmid) or VR1055 (control plasmid), twice per week for three weeks, beginning on day 4 after tumor cell injection (n=8-10 mice per group).

FIG. 4 shows that systemic mIFNα, mIL-2 or mIL-12 plasmid DNA treatment reduces tumor volume (FIG. 4A) and mIFNα or mIL-12 plasmid DNA treatment increases survival (FIG. 4B) in the subcutaneous B16F10 melanoma model. C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected with 100 μg of VR4111 (mIFNα), VR4001 (mIL-12), VR1110 (mIL-2), or VR1012 (control plasmid) (n = 15-16 mice per group) twice per week for three weeks.

FIG. 5 shows that i.m. administration of hIFNω pDNA reduces tumor volume (FIG. 5A) and increases survival (FIG. 5B) in nude mice bearing human A431 epidermoid carcinoma tumors. Mice bearing human A431 tumors between 30-80 mm^3 were injected i.m. with 200 μg of either VR4151 (hIFNω plasmid) or VR1055 (control plasmid) twice per week for three weeks (n=15)

FIG. 6 shows that i.m. administration of mIFNα pDNA reduces B16F10 melanoma lung metastases in C57BL/6 mice. Mice bearing lung

metastases of B16F10 melanoma were injected i.m. with 100 μg of either VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell injection (n=10 mice per group). "TNTC" means too numerous to count as seen in the control group.

FIG. 7 shows that i.m. administration of mIFNα pDNA reduces intradermal M5076 primary tumor growth (FIG. 7A) as well as liver metastases (FIG. 7B) in C57BL/6 mice bearing murine M5076 reticulum cell sarcoma cells. Mice bearing M5076 tumors were injected i.m. with 100 μg of either VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell inoculation (n=10-13 mice per group).

FIG. 8 shows a comparison of different dosages and frequencies of mIFNα pDNA administration in the subcutaneous B16F10 melanoma model. C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected i.m. with 50 μg or 100 μg of either VR4111 or VR1055 twice a week for 3 weeks beginning 4 days after tumor cell inoculation (n=10 mice per group). All groups treated with 100 μg of VR4111 showed significant reduction in tumor growth by day 21 ($p=0.002$) and significant enhancement in survival ($p<0.008$) with all treatments tested (FIGs. 8A and 8B). In mice treated with 50 μg VR4111, tumor growth was significantly reduced by day 21 ($p=0.005$), and survival was significantly increased ($p<0.003$) in the groups of mice that were injected twice per week or once per week. The group injected every other week with 50 μg VR4111 was not significantly different from the mice that received the control plasmid (FIGs. 8C and 8D).

FIG. 9 shows the results of experiments performed to determine the role of NK and T cells in the antitumor response induced by mIFNα plasmid DNA. Nude mice (T cell deficient) (FIGS. 9A and 9B), and beige-nude mice (NK and T cell deficient) (FIGS. 9C and 9D) bearing subcutaneous B16F10 melanoma tumors were injected i.m. with 100 μg of either VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell injection (n=15 mice per group). No significant reduction in tumor volume or increase in survival was found for nude or nude-beige mice treated with

VR4111, suggesting that T cells are involved in the mIFNα antitumor response.

FIG. 10 shows the results of experiments performed to evaluate the role of $CD4^+$ and $CD8^+$ T cells in the mIFNα DNA antitumor response. For depletion of $CD4^+$ and $CD8^+$ T cells, C57BL/6 mice bearing subcutaneous B16F10 melanoma tumors were injected i.p. with 500 μg of either the anti-CD4 mAb (clone GK1.5, rat IgG) (ATCC, Rockville, MD) or anti-CD8 mAb (clone 2.43, rat IgG) (ATCC, Rockville, MD) one day after each i.m. injection of 100 μg of either VR4111 or VR1055 twice per week for three weeks (n = 10 mice per group). The mIFNα plasmid DNA therapy significantly reduced tumor growth ($p \leq 0.002$) and enhanced survival ($p \leq 0.008$) of both normal mice and mice depleted of $CD4^+$ T cells, suggesting that $CD4^+$ T cells were not required for the response. In contrast, mice depleted of $CD8^+$ T cells and injected with VR4111 had tumor volumes and survival that were not significantly different from mice treated with the control plasmid DNA, indicating a requirement for $CD8^+$ T cells in the antitumor response.

FIG. 11 shows that intratumoral hIFNω (VR4151) and hIFNα (VR4112) treatment reduces tumor volume in the human A375 melanoma model (FIG. 11A) and human NIH-OVCAR3 (FIG. 11B) in nude mice. Mice bearing subcutaneous tumor received direct intratumoral injections of a complex of DNA:DMRIE/DOPE (1:1 DNA:lipid mass ratio, 100 μg of plasmid DNA) for 6 consecutive days followed by an additional 5 treatments every other day for a total of 11 injections (A375 melanoma model), or for every other day for a total of 11 injections (NIH-OVCAR3 ovarian cancer model).

FIG. 12 shows that intratumoral mIFNα (VR4101) plasmid DNA treatment reduces tumor volume (FIG. 12A) and increases survival (FIG. 12B) in the subcutaneous B16F10 melanoma model in C57BL/6 mice. Mice received a subcutaneous implantation of 10^4 B16F10 cells into the flank. Beginning at day 12 post tumor implant, mice received six consecutive

intratumoral injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μg of plasmid DNA).

FIG. 13 shows luciferase activity in peritoneal tissues and MOT ascites in mice after i.p. injection of luciferase DNA:lipid complex. The results show high levels of reporter gene expression in ascites but low levels in peritoneal tissue. MOT tumor-bearing C3H/HeN mice received i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μg of plasmid DNA) on days 5 and 6 after tumor cell implant. Tissues were collected 1 day (FIG. 13A) or 3 days (FIG.13B) following the DNA:lipid injection.

FIG. 14 shows serum levels of IL-2 after i.p. injection of either IL-2 pDNA or protein in MOT tumor bearing mice. The serum levels of IL-2 were much lower than levels in ascites. Ascites and serum were collected at 4 hours and days 1, 2, 3, 6 and 10 post DNA or protein injection (5 mice for each time point), and analyzed for mIL-2 polypeptide using an ELISA.

FIG. 15 shows a significant reduction in MOT tumor growth ($p=0.01$) (FIG. 15A) and increased survival ($p=0.04$) (FIG. 15B) of mice treated with i.p. injection of IL-2 pDNA:lipid on days 5-10 after tumor cell injection. The DNA was complexed at either a 1:1 (15A and 15B) or 5:1 (FIGS. 15C and 15D) DNA:DMRIE mass ratio (100 μg pDNA). Plasmid DNA without lipid was not effective (FIGs. 15E and 15F)

FIG. 16 shows that i.p. mIL-2 plasmid DNA (VR1110):lipid treatment inhibits tumor growth (FIG. 16A) and enhances survival (FIG. 16B) in the MOT tumor model in C3H/HeN mice. MOT tumor-bearing mice received three alternative-day i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μg of plasmid DNA).

FIG. 17 shows a significant reduction in MOT tumor growth and increased survival of mice treated with i.p. injection of IL-2 DNA:lipid followed by debulking of tumor ascites. MOT tumor-bearing mice received six consecutive intraperitoneal injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μg of pDNA) and

debulked of 5 ml of tumor ascites 4 days after the last DNA:lipid injection (n=10).

FIG. 18 shows dose-response of mIL-2 pDNA (VR1110):lipid treatment in the MOT tumor model. C3H/HeN mice bearing MOT tumor were injected with 25, 50 or 100 μg of VR1110:DMRIE/DOPE on days 5, 8 and 11 after MOT tumor cell injection. In mice treated with 50 or 100μg of VR1110, tumor growth was significantly reduced (p=0.002) and survival significantly enhanced (p=0.01) by day 15 post tumor cell inoculation compared to the control. Tumor-bearing mice treated with 25 μg of VR1110:lipid were not significantly different from the control mice for either tumor volume or survival (n=15).

FIG. 19 shows the cytokine profile of ovarian tumor ascites in C3H/HeN mice MOT tumor model following mIL-2 pDNA (VR1110):lipid treatment. Mice received i.p. injections of a complex of pDNA/DMRIE/DOPE (1:1 DNA/DMRIE mass ratio, 100 μg of plasmid DNA) on days 5, 8 and 11 after tumor cell implant. Two days after each injection, mice were sacrificed (5 mice for each time point), and the ascites were collected and analyzed for cytokine concentration. The level of IL-2 (days 7, 10 and 13) as well as IFNγ and GM-CSF (days 10 and 13) were markedly elevated suggesting that IL-2 upregulates IFNγ and GM-CSF production.

FIG. 20 shows that i.p. mIFNα pDNA (VR4111):lipid treatment enhances survival (FIG. 20B) in the MOT tumor model in C3H/HeN mice. MOT tumor-bearing mice received three alternative-day i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μg of plasmid DNA).

Detailed Description of the Preferred Embodiments

The present invention is broadly directed to treatment of cancer by administering *in vivo*, into a tissue of a mammal suffering from cancer, at least one polynucleotide construct comprising at least one polynucleotide encoding at least one cytokine, or at least one active fragment thereof. The polynucleotide construct is incorporated into the cells of the mammal *in vivo*, and a therapeutically effective amount of a cytokine is produced *in vivo*, and delivered to tumor cells. Combinations of cytokine-encoding polynucleotides can be administered.

The present invention provides a pharmaceutical composition comprising about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, and wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. The pharmaceutical composition can be used to practice all of the methods of the present invention.

The present invention also provides a pharmaceutical composition obtained by complexing a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that

encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group

consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The pharmaceutical composition of the present invention comprises at least one polynucleotide construct comprising at least one polynucleotide encoding an IFNω, or an active fragment thereof. Preferably, the polynucleotide construct contains a polynucleotide encoding a human IFNω. More preferably, IFNω is encoded by nucleotides 1 to 585 in SEQ ID No. 7 (corresponding to amino acids -23 to 172 in SEQ ID No. 8), or by nucleotides 70 to 585 in SEQ ID No. 7 (corresponding to amino acids 1 to 172 in SEQ ID No. 8). Most preferably, the polynucleotide construct is VR4151 in SEQ ID No. 4. The polynucleotide construct may be complexed with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, the polynucleotide construct is complexed with one or more cationic lipids. More preferably, the polynucleotide construct is complexed with one or more cationic lipids and one or more neutral lipids. Still more preferably, the cationic lipid is (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE) and the neutral lipid is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) such that the mass ratio of polynucleotide construct to lipid is from about 10:1 and about 0.5:1. More preferably, the mass ratio of polynucleotide construct to lipid is from about 5:1 and about 1:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is about 5:1.

Cytokine-encoding plasmids discussed herein include VR4102 (hIFNα in the VR1012 vector) (SEQ ID No. 1), VR4112 (hIFNα in the VR1055 vector) (SEQ ID No. 2), VR4150 (hIFNω in the VR1012 vector) (SEQ ID No. 3), VR4151 (hIFNω in the VR1055 vector) (SEQ ID No. 4), VR4101 (mIFNα in the VR1012 vector) (SEQ ID No. 5), VR4111 (mIFNα in the VR1055 vector) (SEQ ID No. 6), and VR1110 (mIL-2 in the VR1012 vector), VR1103 (hIL-2 in the VR1012 vector) (SEQ ID No: 25), VR4001 (mIL-12 in the VR1033 vector), and VR1700 (mGM-CSF in the VR1012 vector).

Cytokine-encoding cDNAs discussed herein include the cDNA for hIFNω (SEQ ID No. 7), the cDNA for hIFNα (SEQ ID No. 9), the cDNA for mIFNα (SEQ ID No. 11), the cDNA for hIL-2 (SEQ ID No. 13 and the coding portion of SEQ ID No. 25), the cDNA for mIL-2 (for example, as disclosed in Kashima *et al.*, *Nature 313*:402-404 (1985), which is hereby incorporated by reference) the cDNA for mIL-12 (for example, as disclosed in Tone *et al.*, *Eur. J. Immunol. 26*:1222-1227(1996), which is hereby incorporated by reference), and the cDNA for mGM-CSF (for example, as disclosed in Gough *et al.*, *EMBO J. 4*:645-653 (1985), which is hereby incorporated by reference). Cytokine polypeptides discussed herein include hIFNω (SEQ ID No. 8), hIFNα (SEQ ID No. 10), mIFNα (SEQ ID No. 12), and hIL-2 (SEQ ID No. 14 and SEQ ID No. 26).

By "stringent conditions" is intended a hybridization by overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (750 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by repeatedly washing the filters (at least three times) in 0.1x SSC and 0.1% sodium dodecyl sulfate (w/v) for 20 minutes at about 65°C.

By "active fragment" is intended a fragment of a cytokine that displays the antiproliferative activity of the mature or full length cytokine. For example, a full length hIFNω is set forth in amino acids -23 to 172 of SEQ ID No. 8. The corresponding mature hIFNω is set forth in amino acids 1 to 172 of SEQ ID No. 8. Active fragments of hIFNω include, but are not limited to a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, a polypeptide comprising amino acids 61-172 in SEQ ID No. 8, a polypeptide comprising amino acids 41-172 in SEQ ID No. 8, and a polypeptide comprising amino acids 21-172 in SEQ ID No. 8. A full length hIFNα is set forth in amino acids -23 to 166 of SEQ ID No. 10. The corresponding mature hIFNα is set forth in amino acids 1 to 166 of SEQ ID No. 10. Active fragments of hIFNα include, but are not limited to a polypeptide comprising amino acids 83-166 in SEQ ID

No. 10, a polypeptide comprising amino acids 61-166 in SEQ ID No. 10, a polypeptide comprising amino acids 41-166 in SEQ ID No. 10, and a polypeptide comprising amino acids 21-166 in SEQ ID No. 10. Full length hIL-2 is set forth in amino acids -20 to 133 of SEQ ID No. 14. The corresponding mature hIL-2 is set forth in amino acids 1 to133 of SEQ ID No. 14. Active fragments of hIL-2 include, but are not limited to a polypeptide comprising amino acids 58 to 105 in SEQ ID No. 14, and a polypeptide comprising amino acids 20 to 126 in SEQ ID No. 14.

Assays of antiproliferative activity *in vitro* are well known to those of ordinary skill in the art. For example, one antiproliferation assay that can be used is to treat cultured cells, such as human ovarian NIH-OVCAR3 cells (ATCC, Rockville, MD), with supernatants from human melanoma UM449 cells transfected with the polynucleotide construct containing a polynucleotide encoding an IFNω or an active fragment thereof. In this antiproliferation assay, NIH-OVCAR3 cells are cultured and plated in 96 well-tissue culture plates. The plates are incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. Twenty µl of tissue culture supernatants from transfected UM449 cells are added to duplicate wells. An interferon reference standard (*e.g.*, human leukocyte interferon, Sigma Chemical Co., St. Louis, MO) is included in each assay. The cells are incubated with the test samples or the interferon standard for an additional 72 hours at 37°C. To quantitate the effects on cell proliferation, 50 µl of XTT/ECR substrate (Cell Proliferation Kit, Boehringer Mannheim, Indianapolis, IN) is added to each well and the plates are incubated for an additional 24 hours at 37°C prior to measurement of the OD_{490}. Other cell lines can be used in the antiproliferation assay. For example, any of the cells listed on Table 1 can be used. Another antiproliferation assay that can be used is provided in Nieroda, *et al* (*Mol. Cell. Differentiation* 4: 335-351 (1996)).

For treatment of cancer, a polynucleotide construct comprising a polynucleotide encoding a cytokine can be delivered locally, systemically or intra-cavity. In the "systemic delivery" embodiment of the invention, one or more polynucleotide construct comprising one or more polynucleotide

encoding one or more cytokine is administered into a tissue such that the polynucleotide is expressed as the cytokine *in vivo* and the cytokine is released into the circulation, and such that a therapeutically effective amount of the cytokine is systemically delivered to the tumor. In this embodiment, the polynucleotide construct can be administered within *ex vivo* cells or associated with *ex vivo* cellular material. Preferably, the cytokine is an IFNω, IFNα, IFNτ, IFNγ, IFNβ, IL-1, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GM-CSF, or any combination of these, or any combination of one or more of these and one or more additional cytokines. More preferably, the cytokine is an IFNα, IFNω, IL-2, or IL-12. Most preferably, the cytokine is an IFNα or IFNω. Examples of the combination are a polynucleotide encoding an IFNω and an IFNα; a polynucleotide encoding an IFNω and an IL-2; a polynucleotide encoding an IFNα and an IL-2; and a polynucleotide encoding an IFNω, an IFNα, and an IL-2. More preferably, the polynucleotide construct contains a polynucleotide encoding an IFNω and/or an IFNα. Even more preferably, the polynucleotide construct contains a polynucleotide encoding a human IFNω and/or a human IFNα. Even more preferably, the polynucleotide encodes a human IFNω. Preferably, the polynucleotide construct is administered free from *ex vivo* cells and free from *ex vivo* cellular material.

In this embodiment, administration can be into tissue including but not limited to muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph nodes, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, or connective tissue. Preferably, the administration is into muscle tissue, *i.e.*, skeletal muscle, smooth muscle, or myocardium, and the polynucleotide construct is naked. Most preferably, the muscle is skeletal muscle. For polynucleotide constructs in which the polynucleotide encoding a cytokine is DNA, the DNA can be operably linked to a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells.

By "naked" is meant that the polynucleotide construct is free from association with any delivery vehicle known in the art that can act to facilitate

entry into cells, for example, from transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

As used herein, "*ex vivo*" cells are cells into which the polynucleotide construct is introduced, for example, by transfection, lipofection, electroporation, bombardment, or microinjection. The cells containing the polynucleotide construct are then administered *in vivo* into mammalian tissue. Such *ex vivo* polynucleotide constructs are well-known to those of ordinary skill in the art. For example, see Belldegrun, A., *et al.*, *J. Natl. Cancer Inst. 85*: 207-216 (1993); Ferrantini, M. *et al.*, *Cancer Research 53*: 1107-1112 (1993); Ferrantini, M. *et al.*, *J. Immunology* 153: 4604-4615 (1994); Kaido, T., *et al.*, *Int. J. Cancer 60*: 221-229 (1995); Ogura, H., *et al.*, *Cancer Research 50*: 5102-5106 (1990); Santodonato, L., *et al.*, *Human Gene Therapy 7*:1-10 (1996); Santodonato, L., *et al.*, *Gene Therapy 4*:1246-1255 (1997); and Zhang, J.-F. *et al.*, *Cancer Gene Therapy 3*: 31-38 (1996).

The polynucleotide construct is administered in a "cell-free" fashion when it is administered independently, *i.e.*, free of *ex vivo* cells or *ex vivo* cellular material.

In the "local cytokine delivery" embodiment of the present invention, a polynucleotide construct comprising a polynucleotide encoding IFNω and/or IFNα is administered *in vivo* into or near a tumor of a mammal, such that the polynucleotide is incorporated into the cells of the tumor. Tumor cells subsequently express the interferon polypeptide in an amount effective to treat cancer.

In this embodiment, a polynucleotide construct comprising a polynucleotide encoding an IFNω and/or an IFNα can be administered into the tumor. Alternatively, the polynucleotide construct can be administered into non-tumor cells surrounding a tumor, near a tumor, or adjacent to a tumor, such that a therapeutically effective amount of an IFNω and/or an IFNα is produced *in vivo* near or within the tumor and is delivered to the malignant cells of the tumor. One way to provide local delivery of the polynucleotide construct is by administering intravenously a polynucleotide construct

comprising a tumor-targeted promoter, wherein the polynucleotide is incorporated into the cells of the tumor and the cytokine is expressed in the tumor in an amount effective to treat cancer. Preferably, the polynucleotide construct is administered into the tumor.

In the "intra-cavity delivery" embodiment, the present invention provides a method of selectively transfecting malignant cells in a tumor-bearing body cavity of a mammal by introducing a polynucleotide construct into the body cavity, wherein the polynucleotide is incorporated into tumor cells and the tumor cells subsequently express the protein encoded by the polynucleotide in an amount effective to treat cancer. The polynucleotide construct is administered free from *ex vivo* cells and free from *ex vivo* cellular material.

A cavity is a space within the body that can confine a fluid volume for some period of time. The cavity can either be present in a normal animal, or it can be produced as a result of disease, surgery or trauma. Cavities in the normal animal include the peritoneum, the cerebrospinal fluid space, the ventricles of the brain, the plural space around lung, the bronchiolar airways, the nasal sinus, the bladder, the vagina, the ear, the synovium of various joints (knee, hip etc.), the internal network of salivary gland tissue, and the gastrointestinal tract including stomach. Surgical removal of tumor tissue can also produce a space which fits the definition of a cavity. An open wound produced by trauma or surgery and closed by suture can be defined as a cavity, and the area under a blister produced by an infection, abrasion or a burn also fits the definition.

There are special bioavailability considerations when a gene delivery system is administered into a cavity. First the fluid volume in the cavity can be substantially comprised of the vehicle in which the delivery system is suspended. Second, the delivery system can have particular access to cells that are either suspended in the cavity, or that are lining the surface of the cavity. Third, in some cases normally differentiated cells that are lining the cavity may be embedded in an extracellular matrix and, may not be accessible to the delivery system. Thus, the delivery system may preferentially transfect cells

that are growing outside the normal extracellular matrix and avoid the cells that are growing within the extracellular matrix, conferring a kind of cell selectivity to the delivery system.

With respect to the first point, body fluids such as serum, have been shown to inhibit gene delivery systems. For example, the transfection activities of Lipofectin and LipofectAMINE are inhibited by serum. It is thought that serum factors bind to cationic lipid/DNA complexes and block their uptake into cells. In cavity models the endogenous fluid volume can be removed, the cavity can be washed, and the delivery system can be administered into the cavity in a vehicle that is compatible with optimal gene delivery efficacy. Thus the cavity model allows the investigator to create a fluid environment which allows for optimal gene delivery potency.

With respect to the second point, cells that are either floating in the cavity or are lining the surface of the cavity have preferential access to the delivery system and can be preferentially transfected relative to other cells in the body. Since the delivery system is confined within the cavity, peripheral cells in the body outside of the cavity will not be transfected. Thus, there is tissue targeting to the cells within the cavity. For example, gene delivery systems administered into the peritoneal cavity will have access to metastatic tumor cells derived from colon or ovarian cancers that are floating in the peritoneum or are attached to the surfaces of the peritoneum. Delivery systems administered into the plural space should transfect cancer cells in the plural effusion. Delivery systems administered into the cerebral spinal fluid should have access to metastatic cancer cells present there.

With respect to the third point, differentiated cells that are present in normal tissues are often embedded into an extracellular matrix. This matrix can be difficult to penetrate with large particulate delivery systems. Some cells, such as poorly differentiated tumor cells, that are present in cavities can grow outside of the normal extracellular matrix and are therefore more accessible to gene delivery systems. In this way the delivery system can preferentially transfect those cells that are growing outside of the extracellular matrix and not transfect those cells that are growing within the extracellular

matrix. This is another form of in vivo, cell type specific targeting. Examples of normal cells that are not embedded in an extracellular matrix and are therefore more accessible to gene delivery systems are, bronchial airway cells, lung cells in the plural space, and ependimal cells lining the surface of the ventricles of the brain. Normal bladder cells that line the surface of the bladder are embedded in a tight extracellular matrix and are therefore not readily accessible to a gene delivery system delivered into the bladder, but tumor cells which grow up and out of the extracellular matrix into the bladder vesicle are accessible to gene delivery systems administered into the bladder vesicle. Thus normal bladder tissue would be expected to resist transfection whereas, bladder tumor would be expected to be transfectable.

A preferred application of the intra-cavity delivery embodiment is in the treatment of peritoneally disseminated cancers. More specifically, a mammal bearing peritoneal tumor may be injected i.p. with an effective amount of a polynucleotide complexed with a lipid in a physiologically acceptable diluent in a total volume sufficient to access the entire body cavity. The mammal may have tumor ascites in the peritoneal cavity as in an ovarian cancer. In the most preferred application, this methodology may be used in treating ovarian cancer of a human.

Debulking of tumor ascites is commonly performed on human ovarian cancer patients. Debulking involves removal of tumor ascites from the peritoneal cavity. In humans bearing ovarian tumor ascites, the ascites fluid would be debulked by insertion of a catheter i.p. followed by periodic draining of ascites fluid. It is contemplated that the tumor ascites would be debulked before and/or after the i.p. administration of the polynucleotide formulation of the present invention.

Transfection efficacy of the intra-cavity delivery embodiment may be determined by collecting the tumor ascites and serum at various times after the injection and performing diagnostic assays appropriate for the encoded molecule(s). Naturally, other means of determining tumor mass, growth, and viability may also be used to assess the effectiveness of the present invention.

Preferred polynucleotides for the intra-cavity delivery embodiment may encode not only immunogenic molecules such as cytokines (*e.g.*, interleukins 1-18 and α/β/γ/ω -interferons, colony stimulating factors, *e.g.*, G-CSF, GM-CSF, M-CSF, and tumor necrosis factors), but also chemokines (*e.g.*, C-X-C and C-C), Class I and II histocompatibility antigens, costimulatory molecules (*e.g.*, B7-1, B7-2, CAMs, and flt3 ligand), growth factors (*e.g.*, epidermal growth factors, fibroblast growth factors, transforming growth factors and growth hormone), and the like. The polynucleotide may also encode bacterial antigens, viral glycoproteins, enzymes (*e.g.*, lysozymes), recombinant antibodies, molecules that interfere with cellular adhesion, adhesion molecules, proliferation and vascular inhibitory factors, ribozymes, and antisense RNAs targeted toward key oncogenic or tumor growth proteins. Moreover, selective delivery of toxic peptides (*e.g.*, ricin, diphtheria toxin, or cobra venom factor) or proteins capable of synthesizing toxic compounds (*e.g.* thymidine kinase and cytosine deaminase) to the malignant cells may have therapeutic benefits. The polynucleotide may also comprise a tumor suppressor gene (*e.g.*, p53). Preferred polynucleotides encode cytokines. Preferred cytokines are IL-2, IFNω, and IFNα. IL-2 is most preferred.

For treatment of cancer by any of the above disclosed embodiments, any polynucleotide encoding an IFNω, or an active fragment thereof, can be used. For example, the polynucleotide construct can be a construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when

added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, IFNω is encoded by nucleotides 1 to 585 in SEQ ID No. 7 (corresponding to amino acids -23 to 172 in SEQ ID No. 8), or by nucleotides 70 to 585 in SEQ ID No. 7 (corresponding to amino acids 1 to 172 in SEQ ID No. 8). More preferably, the polynucleotide construct is VR4151.

For treatment of cancer, any polynucleotide encoding IFNα, or active fragment thereof, can also be used. For example, the polynucleotide construct can be a construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 9 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 166 or 1 to 166 in SEQ ID No. 10, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising amino acids 83-166 in SEQ ID No. 10, wherein the polypeptide has antiproliferative activity when

added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, IFNα is encoded by nucleotides 1 to 567 in SEQ ID No. 9 (corresponding to amino acids -23 to 166 in SEQ ID No. 10), or by nucleotides 1 to 567 in SEQ ID No. 9 (corresponding to amino acids 1 to 166 in SEQ ID No. 10). Preferably, the polynucleotide construct is VR4112.

For polynucleotide constructs that do not contain a polynucleotide encoding IFNω, the polynucleotide construct is preferably a cell-free construct. For polynucleotide constructs that contain a polynucleotide encoding IFNω, the polynucleotide construct can be administered either within *ex vivo* cells or free of *ex vivo* cells or *ex vivo* cellular material. Preferably, the polynucleotide construct is administered free of *ex vivo* cells or *ex vivo* cellular material.

In the "local delivery" and "intra-cavity delivery" embodiments, the polynucleotide construct is preferably complexed with one or more cationic compounds. More preferably, the polynucleotide construct is complexed with one or more cationic lipids by ionic interaction. Generally, the complex then contacts the cell membrane and is transfected into the cell. This transfection mechanism is referred to as "lipofection," and is a highly efficient transfection procedure (Felgner, *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1987); and Felgner, *et al., Nature 337*:387-388, 1989). Still more preferably, the polynucleotide construct is complexed with one or more cationic lipids and one or more neutral lipids.

For purposes of the present invention, lipid refers to a synthetic or naturally occurring compound that possesses both a lipophilic region and a polar region, commonly referred to as a head group. Preferred cationic compounds are cationic lipids. Cationic lipids are described in U.S. Pat. Nos. 4,897,355; 4,946,787; 5,049,386; 5,264,618; 5,279,833; 5,334,761; 5,429,127; 5,459,127; 5,589,466; 5,676,954; 5,693,622; 5,580,859; 5,703,055; and 5,578,475; and international publications WO 04/9469, WO 95/14381, 95/14651, 95/17373, 96/18372, 96/26179, 96/40962, 96/40963, 96/41873, and

97/00241, and documents cited therein. As illustrated in the above-cited patents and patent applications, cationic lipids comprise structural features that may be present in a variety of core molecular classes.

Examples of cationic lipids are 5-carboxyspermylglycine dioctadecylamide (DOGS) and dipalmitoyl-phophatidylethanolamine-5-carboxyspermylamide (DPPES). Cationic cholesterol derivatives are also useful, including {3β-[N-N',N'-dimethylamino)ethane]-carbomoyl}-cholesterol (DC-Chol). Dimethyldioctdecyl-ammonium bromide (DDAB), N-(3-aminopropyl)-N,N-(*bis*-(2-tetradecyloxyethyl))-N-methyl-ammonium bromide (PA-DEMO), N-(3-aminopropyl)-N,N-(*bis*-(2-dodecyloxyethyl))-N-methyl-ammonium bromide (PA-DELO), N,N,N-*tris*-(2-dodecyloxy)ethyl-N-(3-amino)propyl-ammonium bromide (PA-TELO), and N^1-(3-aminopropyl)((2-dodecyloxy)ethyl)-N^2-(2-dodecyloxy)ethyl-1-piperazinaminium bromide (GA-LOE-BP) can also be employed in the present invention.

Non-diether cationic lipids, such as DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium (DORI diester), 1-O-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium (DORI ester/ether), and their salts promote *in vivo* gene delivery. Preferred cationic lipids comprise groups attached via a heteroatom attached to the quaternary ammonium moiety in the head group. A glycyl spacer can connect the linker to the hydroxyl group.

Preferred cationic lipids are 3,5-(N,N-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) (DLYS-DABA-DORI diester), 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) (DLYS-DABA-GLY-DORI diester), and (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE).

Also preferred are (±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA), (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (β-aminoethyl-DMRIE or βAE-DMRIE) (Wheeler, *et al.*, *Biochim. Biophys. Acta 1280*:1-11 (1996)), and (±)-

N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide (GAP-DLRIE) (Wheeler, *et al., Proc. Natl. Acad. Sci. USA* 93:11454-11459 (1996)), which have been developed from DMRIE.

Other examples of DMRIE-derived cationic lipids that are useful for the present invention are (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-decyloxy)-1-propanaminium bromide (GAP-DDRIE), (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(*bis*-tetradecyloxy)-1-propanaminium bromide (GAP-DMRIE), (±)-N-((N"-methyl)-N'-ureyl)propyl-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (GMU-DMRIE), (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (DLRIE), and (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-*bis*-([Z]-9-octadecenyloxy)propyl-1- propaniminium bromide (HP-DORIE).

The lipids of the lipid-containing formulation can comprise a cationic lipid alone, or further comprise a neutral lipid such as cardiolipin, phosphatidylcholine, phosphatidylethanolamine, dioleoylphosphatidylcholine, dioleoylphosphatidyl-ethanolamine,1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), sphingomyelin, and mono-, di- or tri-acylglycerol. Other additives, such as cholesterol, fatty acid, ganglioside, glycolipid, neobee, niosome, prostaglandin, sphingolipid, and any other natural or synthetic amphiphiles, can also be used. A preferred molar ratio of cationic lipid to neutral lipid in these lipid-containing formulations is from about 9:1 to about 1:9; an equimolar ratio is particularly preferred. The lipid-containing formulation can further comprise a lyso lipid (*e.g.*, lyso-phosphatidylcholine, lysophosphatidyl-ethanolamine, or a lyso form of a cationic lipid).

More preferably, the cationic lipid is (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE) and the neutral lipid is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) such that the mass ratio of polynucleotide construct to lipid is from about 10:1 and about 0.5:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is from about 5:1 and about 1:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is about 5:1.

Lipid-containing pharmaceutical composition for use in a complex with the polynucleotide construct of the present invention can also comprise cationic lipid together with an effective amount of a lysophosphatide. The lysophosphatide can have a neutral or a negative head group. Lysophosphatidylcholine and lysophosphatidyl-ethanolamine are preferred, and 1-oleoyl lysophosphatidylcholine is particularly preferred. Lysophosphatide lipids are advantageously present in the lipid-containing formulation in a 1:2 ratio of lysolipid to cationic lipid. Lyso forms of a cationic lipid can also be used to promote polynucleotide delivery. These lyso forms are advantageously present in effective amounts up to about one-third of the total cationic lipid in the lipid-containing formulations.

In a formulation for preparing DNA: lipid complexes, the cationic lipid can be present at a concentration of between about 0.1 mole % and about 100 mole %, preferably about 5 mole % and 100 mole %, and most preferably between about 20 mole % and 100 mole %, relative to other formulation components present in the formulation. The neutral lipid can be present in a concentration of between zero and about 99.9 mole %, preferably zero and about 95 mole %, and most preferably zero and about 80 mole %. In order to produce lipid vesicles having a net positive charge, the quantity of the positively charged component must exceed that of the negatively charged component. The negatively charged lipid can be present at between zero and about 49 mole %, and preferably between zero and about 40 mole %. Cholesterol or a similar sterol can be present at between zero to about 80 mole %, and preferably zero and about 50 mole %.

The polynucleotide to be delivered can be solubilized in a buffer prior to mixing with lipid vesicles. Suitable buffers include, for example, phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate vehicle (100-150 mM preferred). Insoluble polynucleotides can be solubilized in a weak acid or base, and then diluted to the desired volume with a neutral buffer such as PBS. The pH of the buffer is suitably adjusted, and moreover, a pharmaceutically acceptable additive can be used in the buffer to provide an appropriate osmolarity within the lipid vesicle.

A lipid solution comprising at least one amphipathic lipid can spontaneously assemble to form primary lipid vesicles, heterogeneous in size. Therefore, according to a preferred method, the lipids of the lipid-containing formulation, comprising at least one cationic lipid, are prepared by dissolution in a solvent such as chloroform and the mixture is evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary lipid vesicles. These primary lipid vesicles may be reduced to a selected mean diameter by means of a freeze-thaw procedure. Vesicles of uniform size can be formed prior to drug delivery according to methods for vesicle production known to those in the art; for example, the sonication of a lipid solution as described by Felgner, *et al* (*Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987)) and U.S. Pat. No. 5,264,618.

The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to humans; primate mammals such as apes, monkeys, orangutans, and chimpanzees; canine mammals such as dogs and wolves; feline mammals such as cats, lions, and tigers; equine mammals such as horses, donkeys, deer, zebra, and giraffe; and bears. Preferably, the mammal is a human subject.

Tumor cell formation and growth, also known as "transformation," describes the formation and proliferation of cells that have lost their ability to control cellular division; that is the cells are cancerous. "Malignant cells" are defined as cells that have lost the ability to control the cell division cycle, leading to a transformed or cancerous phenotype.

The term "non-tumor tissue" is intended to include, but is not limited to non-tumor bearing tissues such as muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, or connective tissue. Preferably, the non-tumor tissue is muscle.

Preferably, the polynucleotide construct is delivered to the interstitial space of a tumor or of non-tumor tissues. "Interstitial space" comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of

organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels.

The pharmaceutical composition and methods of the present invention can be used to treat a variety of mammalian cancers or tumors. Types of mammalian cancers and tumors that can be treated using the pharmaceutical composition and methods of the present invention include, but are not limited to all solid tumors, cutaneous tumors, melanoma, malignant melanoma, renal cell carcinoma, colorectal carcinoma, colon cancer, hepatic metastases of advanced colorectal carcinoma, lymphomas (including glandular lymphoma), malignant lymphoma, Kaposi's sarcoma, prostate cancer, kidney cancer, ovarian cancer, lung cancer, head and neck cancer, pancreatic cancer, mesenteric cancer, gastric cancer, rectal cancer, stomach cancer, bladder cancer, leukemia (including hairy cell leukemia and chronic myelogenous leukemia), breast cancer, non-melanoma skin cancer (including squamous cell carcinoma and basal cell carcinoma), hemangioma multiple myeloma, and glioma. Preferably, the cancer is melanoma, ovarian cancer, or metastases thereof.

By "treatment" is meant reduction in tumor size, a reduction in the rate of metastasis, and/or a slowing of tumor growth, and/or no worsening in disease over a specified period of time.

A systemic delivery embodiment can be particularly useful for treating nonlocalized tumors (*i.e.*, leukemia and metastases of a variety of tumors), or a disease category that might be responsive to continuous exposure by the systemic route (i.e., myeloma, chronic myelogenous leukemia, lymphoma). A local delivery embodiment can be particularly useful for treating one disease condition that might be responsive to high local concentration (i.e., renal cell carcinoma, melanoma). For tumors involving body cavity of a mammal, "intra-cavity" embodiment is preferred. In particular, the use of this methodology is envisioned in treating cancers involving (i) the peritoneal cavity--pancreatic cancer, gastric cancer, ovarian cancer, mesenteric cancer,

glandular lymphoma and metastatic melanoma; (ii) the thoracic cavity--lung cancer and glandular lymphoma; (iii) the rectal cavity--rectal cancer; (iv) the stomach cavity--stomach cancer; and (v) the urinary bladder vesicle--bladder cancer. When advantageous, systemic, local, and/or intra-cavity delivery can be combined, especially in a mammal having a primary site of tumor and one or more metastases.

An additional embodiment of the present invention is directed to combining any of the methods of the present invention with one or more additional cancer therapies including, but not limited to bone marrow transplant, cord blood cell transplant, surgery, chemotherapy, radiation therapy, and immunotherapy. The polynucleotide construct or pharmaceutical composition of the present invention can be administered prior to the commencement of one or more of the additional cancer therapies, during the practice of one or more of the additional cancer therapies, and after the end of one or more of the additional cancer therapies.

Types of bone marrow transplant include, but are not limited to autologous bone marrow transplant and heterologous (*i.e.*, from a donor) bone marrow transplant.

Types of surgery include, but are not limited to surgery for breast cancer, prostate cancer, colon cancer, brain cancer, and head and neck cancer.

Chemotherapeutic agents include, but are not limited to alkylating agents, including mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, dicarbazine, streptazocine, carmustine, lomustine, semustine, chlorozotocin, busulfan, triethylenemelamine, thiotepa, hexamethylmelamine; antimetabolites, including methotrexate; pyrimidine analogs, including fluorouracil, 5-fluorouracil, floxuridine (5'-fluoro-2'-deoxyuridine), idoxuridine, cytarabine, –phosphonoacetyl-L-aspartate, 5-azacytidine, azaribine, 6-azauridine, pyrazofuran, 3-deazauridine, acivicin; purine analogs, including thioguanine, mercaptopurine, azathioprine, pentostatin, erythrohydroxynonyladenine; vinca alkaloids, including vincristine and vinblastine; epipodophyllotoxins, including etoposide and teniposide; antibiotics, including dactinomycin, daunorubicin, doxorubicin,

bleomycin sulfate, plicamycin, mitomycin; enzymes, including L-asparaginase; platinum coordination complexes, including cisplatin, carboplatin; hydroxyurea, procarbazine, mitotane; and hormones or related agents, including adrenocorticosteroids such as prednisone and prednisolone; aminoglutethimide; progestins such as hydroxyprogesterone caproate, medroxyprogesterone acetate, megesterol acetate, estrogens and androgens such as diethylstilbestrol, fluoxymesterone, ethynyl estradiol, antiestrogens such as tamoxifen, and gonadotropin-releasing hormone analogs such as leuprolide.

The present invention also provides kits for use in treating cancer comprising an administration means and a container means containing one or more cytokine-expressing polynucleotide constructs in a sterile environment. Also provided are kits for use in treating cancer comprising an administration means and a container means containing one or more cytokine-expressing polynucleotide constructs and one or more cationic compounds in a sterile environment. Examples of cationic compounds are described above. The cytokine-expressing polynucleotide constructs and the cationic compounds may be in the same container means or in separate container means. Preferably, the polynucleotide construct is in the amount of 1 ng to 20 mg.

Cotainer means include glass containers, plastic containers, or strips of plastic or paper. In one embodiment, the container means is a syringe and the administration means is a plunger. In another embodiment, the administration means is a catheter.

The cytokine encoded by the polynucleotide construct of the kit of the present invention can be an IFNω and one or more additional cytokines, including any of the cytokines described herein. Preferably, the cytokine is IFNω and/or an IFNα. The construct can be in the form of a pharmaceutical composition and can contain a pharmaceutically acceptable carrier. Pharmaceutical compositions are described above. The kit can further comprise a pharmaceutically acceptable carrier in a separate container means.

The kit can further comprise an instruction sheet for administration of the composition into a mammal. The components of the polynucleotide

composition are preferably provided as a liquid solution, such as a suspension, a solution, or an emulsion; or in lypholized form as a dried powder or a cake. If the polynucleotide construct is provided in lypholized form, preferably the kit further comprises a container means containing a suitable vehicle, such as sterile pyrogen-free water, for reconstitution of the lypholized polynucleotide construct, or any buffer described herein, including PBS, normal saline, Tris buffer, and sodium phosphate vehicle.

The term "cytokine" refers to polypeptides, including but not limited to interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, and IL-18), α interferons (e.g, IFNα), β interferons (*e.g.*, IFNβ), γ interferons (*e.g.*, IFNγ), ω interferon (IFNω), τ interferons (IFNτ), colony stimulating factors (CSFs, *e.g.*, CSF-1, CSF-2, and CSF-3), granulocyte-macrophage colony stimulating factor (GMCSF), epidermal growth factor (EGF), fibroblast growth factors (FGFs, *e.g.*, acidic fibroblast growth factor, basic fibroblast growth factor, FGF-1, FGF-2, FGF-3, FGF-4, and FGF-5), transforming growth factor (TGF, *e.g.*,.TGFα and TGFβ), platelet-derived growth factor (PDGF), tumor necrosis factors (TNFs, *e.g.*, TNF-α and TNF-β), and insulin-like growth factors (IGFs, e.g, IGF-I and IGF-II).

A "polypeptide" refers to any translation product of a polynucleotide, regardless of the size of the translation product, and regardless of whether the translation product is post-translationally modified (*e.g.*, glycosylated) or not.

The polynucleotide construct of the present invention, whether complexed with cationic vehicle or not, can be administered by any suitable route of administration, including intramuscularly, subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane). Similarly, the pharmaceutical composition of the present invention can by administered by any suitable route, including intramuscularly, into or near a tumor, into a cavity (*e.g.*, intraperitoneally), subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane).

Any mode of administration can be used so long as the mode results in the expression of one or more cytokines in an amount sufficient to decrease the tumorigenicity of the cancer bearing mammal. This includes needle injection, catheter infusion, biolistic injectors, particle accelerators (*i.e.*, "gene guns"), pneumatic "needleless" injectors (*e.g.*, MedEJet, PedoJet, Bioject), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (*e.g.*, Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. Preferred methods include needle injection and catheter infusion.

A "polynucleotide construct" is a polynucleotide molecule that carries genetic information for encoding one or more molecules, preferably, cytokines. The polynucleotide material delivered to the cells *in vivo* can take any number of forms. It can contain the entire sequence or only a functionally active fragment of a cytokine gene.

The polynucleotide construct comprises at least one polynucleotide (*e.g.*, DNA, RNA, ribozyme, phosphorothioate, or other modified nucleic acid) encoding one or more molecules. Preferred molecules are cytokines. The polynucleotide can be provided in linear, circular (*e.g.* plasmid), or branched form; and double-stranded or single-stranded form. The polynucleotide can involve a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond as in peptide nucleic acid (PNA)). The choice of polynucleotide encoding a cytokine will depend on the desired kinetics and duration of expression. When long term delivery of the polynucleotide construct is desired, the preferred polynucleotide is DNA. Alternatively, when short term delivery is desired, the preferred polynucleotide is mRNA. RNA will be rapidly translated into polypeptide, but will be degraded by the target cell more quickly than DNA. In general, because of the greater resistance of circular DNA molecules to nucleases, circular DNA molecules will persist longer than linear polynucleotides, and they will be less likely to cause insertional mutation by integrating into the target genome.

In one embodiment, the polynucleotide sequence encoding one or more cytokines is RNA. Most preferably, the RNA is messenger RNA (mRNA). Methods for introducing RNA sequences into mammalian cells is described in U.S. patent No. 5,580,859. A viral alphavector, a non-infectious vector useful for administering RNA, may be used to introduce RNA into mammalian cells. Methods for the *in vivo* introduction of alphaviral vectors to mammalian tissues are described in Altman-Hamamdzic, S., *et al.*, *Gene Therapy 4*: 815-822 (1997). Preferably, the polynucleotide sequence encoding one or more cytokines is DNA. In a DNA construct, a promoter is preferably operably linked to the polynucleotide encoding a cytokine. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, can be included in the polynucleotide construct to direct cell-specific transcription of the DNA.

An operable linkage is a linkage in which a polynucleotide sequence encoding a cytokine is connected to one or more regulatory sequence in such a way as to place expression of the cytokine sequence under the influence or control of the regulatory sequence(s). Two DNA sequences (such as a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence) are operably linked if induction of promoter function results in the transcription of mRNA encoding the desired polypeptide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the polypeptide, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of affecting transcription of that DNA sequence.

Preferably, the polynucleotide construct is a circular or linearized plasmid containing non-infectious nucleotide sequence. A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. The polynucleotide sequence

encoding a cytokine may comprise a sequence which directs the secretion of the polypeptide.

"Non-infectious" means that the polynucleotide construct does not infect mammalian cells. Thus, the polynucleotide construct can contain functional sequences from non-mammalian (*e.g.*, viral or bacterial) species, but the construct does not contain non-mammalian nucleotide sequences that facilitate infection of the construct into mammalian cells.

"Non-integrating" means that the polynucleotide construct does not integrate into the genome of mammalian cells. The construct can be a non-replicating DNA sequence, or specific replicating sequences genetically engineered to lack the ability to integrate into the genome. The polynucleotide construct does not contain functional sequences that facilitate integration of the cytokine-encoding polynucleotide sequence into the genome of mammalian cells.

The polynucleotide construct is assembled out of components where different selectable genes, origins, promoters, introns, 5' untranslated (UT) sequence, terminators, polyadenylation signals, 3' UT sequence, and leader peptides, etc. are put together to make the desired vector. The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

The polynucleotide construct can be an expression vector. A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing.

Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from retroviruses, *e.g.*, RSV, HTLVI, HIVI, MPSV and the immediate early promoter of the cytomegalovirus (CMV IEP). However, cellular elements can also be used (*e.g.*, the human actin promoter, metallothionein promoter). In humans, CMV IEP is preferred. Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109), VR1012, VR1055, and pcDNA3 (Invitrogen, San Diego, CA). All forms of DNA, whether replicating or non-replicating, which do not become integrated into the genome, and which are expressible, are within the methods contemplated by the invention.

The vector containing the DNA sequence (or the corresponding RNA sequence) which can be used in accordance with the invention can be a eukaryotic expression vector. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman, *et al., Proc. Nat. Acad. Sci. (USA) 84*:2150-2154 (1987).

Secretion of a cytokine from a cell can be facilitated by a leader or secretory signal sequence. In a preferred embodiment, either the native leader sequence of a cytokine is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the peptide that is operably linked to it. Alternatively, a heterologous mammalian leader sequence, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator or mouse β-glucuronidase.

For the methods of the present invention, a single polynucleotide construct containing more than one polynucleotide sequences encoding one or more molecules, or more than one polynucleotide constructs each containing polynucleotide sequences encoding one or more molecules may be co-injected or sequentially injected. For example, a single polynucleotide construct containing one polynucleotide encoding an interferon and another polynucleotide encoding an additional cytokine or an immunomodulatory

molecule, *i.e.*, MHC class I antigen, tumor antigen, and co-stimulatory molecule, can be injected. Alternatively, two polynucleotide construct can be injected where one encodes a cytokine to enhance anti-tumor efficacy of the other gene product. For example, an IFNω, IFNα, IL-12 or IL-2-expressing polynucleotide construct can be co-injected with a polynucleotide construct encoding a different cytokine. More specifically, an IL-2 expressing plasmid could be co-injected with a G-CSF or GM-CSF expressing plasmid. Alternatively, one or more plasmids could be administered initially and other plasmid(s) could be administered subsequently at various time intervals. Combination of the present invention with therapeutic agents such as lymphokine-activated killer cells (LAK) and tumor-infiltrating lymphocytes (TIL) is also envisioned.

It will be recognized in the art that some amino acid sequences of the polypeptides described herein can be varied without significant effect on the functional activity of the polypeptides. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the polypeptide which determine activity. Such variations include deletions, insertions, inversions, repeats, and type substitutions. Guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," (*Science 247*:1306-1310 (1990)). Compositions within the scope of the invention can be assayed according to the antiproliferation assay described herein. Amino acids that are critical for cytokine activity can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos, *et al. Science 255*:306-312 (1992)).

The present invention further relates to using variants of the cytokine-encoding polynucleotide, which encode portions, analogs or derivatives of the cytokine. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John

Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the cytokine or portions thereof. Also especially preferred in this regard are conservative substitutions. For example, aromatic amino acids that can be conservatively substituted for one another include phenylalanine, tryptophan, and tyrosine. Hydrophobic amino acids that can be conservatively substituted for one another include leucine, isoleucine, and valine. Polar amino acids that can be conservatively substituted for one another include glutamine and asparagine. Basic amino acids that can be conservatively substituted for one another include arginine, lysine, and histidine. Acidic amino acids that can be conservatively substituted for one another include aspartic acid and glutamic acid. Small amino acids that can be conservatively substituted for one another include alanine, serine, threonine, methionine, and glycine.

Substitutions, deletions, or insertions can be made outside of the region encoding the shortest active fragment of the cytokine, without affecting the activity of the cytokine. Further, mutated proteins (or muteins) often retain a biological activity that is similar to that of the naturally occurring protein. For example, Gayle and coworkers (*J. Biol. Chem. 268:* 22105-22111 (1993)) conducted an extensive mutational analysis of the human cytokine IL-1α. They used random mutagenesis to generate over 3,500 individual IL-1α mutants with an average of 2.5 amino acid changes per mutein over the entire length of the molecule. Multiple mutations were examined at every possible amino acid and, on average, each mutein's amino acid sequence was 98.4% identical to that of naturally occurring IL-1α. The investigators observed that most of the molecule could be mutated with little effect on either binding or biological activity, and that 75% of the molecule may not contribute significantly to the biological activity of the molecule.

Similarly, Gronenborn and colleagues (*FEBS Letters 231*: 135-138 (1988)) analyzed the receptor binding activity of six mutant IL-1α polypeptides. Each mutant contained a single amino acid alteration from the naturally occurring IL-1α polypeptide and was examined under four sets of experimental conditions. In this study, the investigators found very little difference between the receptor binding activity of the mutants and naturally occurring IL-1α.

Further, Zurawski and colleagues (*EMBO J. 12*: 5113-5119 (1993)) studied residues 41-142 of mIL-2 by generating 1,090 muteins. The extent of the mutagenesis was such that there was an average of 11 different amino acid substitutions per naturally occurring amino acid residue, with the exception of the extreme N– and C-termini and residues 31-40. The mIL-2 muteins were assayed for specific activity and compared to that of naturally occurring mIL-2. The degree to which the specific activity was antagonized by a previously characterized mIL-2 mutant was also assessed. The investigators observed that in the 149 residue mIL-2 protein, only 23 residues are important for interaction with IL-2R, 18 residues are presumed to be part of the structural core, and three additional residues are important for structure. 98 mIL-2 residues (or 65% of the protein) were assigned as relatively unimportant residues.

The polynucleotide and amino acid sequences encoding an IFNω include the sequences for the complete IFNω and the mature IFNω set forth in U.S. Patent No. 4,917,887; European Patent Publication No. 0 170 204 B1; and Capon, D.J., *et al., Molec. Cell. Biol. 5*: 768-779 (1985); Hauptmann, R. and P. Swetly, *Nucl. Acids Res.* 13: 4739-4749 (1985); Adolf, G.R., *et al., Biochim. Biophys. Acta 1089*: 167-174 (1991); Mege, D., *et al., J. Interf. Res. 11*: 341-350 (1991); Charlier, M., *et al., J. Interf. Res. 13* 313-322 (1993); Hughes, A.L., *J. Mol. Evol. 41*: 539-548 (1995); and Roberts, R.M., *et al., Prog. Nucl. Acid Res. Molec. Biol. 56*:287-325, edited by W.E. Cohn, Academic Press (1997).

The polynucleotide and amino acid sequences encoding IFNα include the sequences for the complete IFNα and the mature IFNα set forth in U.S. Patent Nos. 4,530,901; 4,695,543; 4,695,623; 4,748,233; 4,892,743; 4,897,471; 4,973,479; 4,975,276; and 5,098,703; and in Pestka, S., *Methods Enzymol. 119*: 3-14 (1986); Hughes, A.L., *J. Mol. Evol. 41*: 539-548 (1995); and Roberts, R.M., *et al., Prog. Nucl. Acid Res. Molec. Biol. 56*:287-325, edited by W.E. Cohn, Academic Press (1997).

The polynucleotide and amino acid sequences encoding an IL-2 include the sequences for the complete human IL-2 and mature IL-2 set forth in Lupker, J. *et al.*, EP 0307285-A3 (1989), U.S. Patent No. 5,641,665, Maeda *et al., Biochem. Biophys. Res. Commun. 115*:1040-1047 (1983), Mita *et al., Biochem. Biophys. Res. Commun. 117*:114-121 (1983), Taniguchi *et al., Nature 302*:305-310 (1983), Devos *et al., Nucleic Acid Res. 11*:4307-4323 (1983), Fujita *et al., Proc. Natl. Acad. Sci. USA 80*:74347-7441 (1983), Clark *et al., Proc. Natl. Acad. Sci. USA 81*:2543-2547 (1984) and Cullen, *DNA 7*:645-650 (1988).

The polynucleotide sequences encoding an IFNω, an IFNα, and an IL-2 also include sequences that encode the complete polypeptide encoded by the nucleotide sequences set forth in SEQ ID Nos. 7, 9 and 13, respectively, and the mature polypeptides encoded by nucleotide sequences set forth in SEQ ID Nos. 7, 9 and 13, respectively. The polynucleotide sequences encoding IL-2 further includes the sequence that encodes the complete IL-2 polypeptide encoded by the nucleotide sequence set forth in SEQ ID No. 25, shown as SEQ ID No. 26.

Thus, a polynucleotide sequence encoding a polypeptide of the present invention can encode a polypeptide having one to twenty amino acid substitutions, deletions or insertions, either from natural mutations or human manipulation, relative to the full length or mature IFNα, IFNω, or IL-2. Preferably, no more than one to fifteen substitutions, deletions or insertions are present, relative to the full length or mature IFNα, IFNω, or IL-2 (excluding the signal sequence). More preferably, no more than one to ten

substitutions, deletions or insertions are present. Still more preferably, no more than one to five substitutions, deletions or insertions are present.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the mammal, the precise condition requiring treatment and its severity, and the route of administration. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

If the polynucleotide construct of the present invention is administered as a pharmaceutical composition, the pharmaceutical composition can be formulated according to known methods for preparing pharmaceutical compositions, whereby the substance to be delivered is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in Remington's Pharmaceutical Sciences, 16th Edition, A. Osol, Ed., Mack Publishing Co., Easton, PA (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA (1995).

The pharmaceutical composition can be in the form of an emulsion, gel, solution, suspension, or other form known in the art. Optionally, it can contain one or more lipids as described above. In addition, the pharmaceutical composition can also contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives. Administration of pharmaceutically acceptable salts of the polynucleotides described herein is preferred. Such salts can be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

For aqueous pharmaceutical compositions used *in vivo*, sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of the substance together with a suitable amount of vehicle in order to

prepare pharmaceutically acceptable compositions suitable for administration to a human or animal.

A pharmaceutical composition can be in solution form, or alternatively, in lyophilized form for reconstitution with a suitable vehicle, such as sterile, pyrogen-free water. Both liquid and lyophilized forms will comprise one or more agents, preferably buffers, in amounts necessary to suitably adjust the pH of the injected solution.

The container in which the pharmaceutical formulation is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The pharmaceutical formulation is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation until use. Optionally, the container can be associated with administration means and or instruction for use.

Having now generally described the invention, the same will become more readily understood by reference to the following specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Described herein are: 1) the *in vitro* characterization of biological activities of and IFNs delivered by plasmid DNA (anti-proliferative activity and anti-viral activity *in vitro*); 2) *in vivo* expression of cytokines following *in vivo* administration of cytokine-expressing pDNA; and 3) the *in vivo* characterization of anti-tumor activity of cytokines in murine models of solid and metastatic tumors following intratumoral, intramuscular or intra-cavity administration of cytokine-encoding pDNA.

The cytokine-encoding polynucleotide constructs have potent anti-proliferative activity *in vitro*. Moreover, the *in vivo* anti-tumor activities of IFNω, IFNα, IL-2, and IL-12 are herein demonstrated in multiple murine tumor models including nude mice bearing subcutaneous human tumors, or in immunocompetent mice bearing murine solid and metastatic tumors. Intratumoral, intramuscular, or intraperitoneal injection of the cytokine-

encoding plasmids is shown to result in a statistically significant slowing of tumor growth and/or a statistically significant increase in survival. In addition to the potent antitumor effects of the cytokine plasmids delivered via intratumoral or intramuscular injection, this is the first *in vivo* demonstration of anti-tumor activity for human interferon-ω. Moreover, the *in vivo* antitumor activity of IL-2 in the treatment of peritoneally disseminated cancers, such as ovarian metastatic melanoma is also demonstrated.

Example 1

Construction of Expression Vectors

Three basic eukaryotic expression plasmid vectors, termed VR1012, VR1055 and VR1033 were used in the construction of all plasmids used in the following examples. The blank plasmids, VR1012 and VR1055 differ only in transcriptional termination sequences. The backbone of both plasmids is derived from pUC19, with the beta-lactamase (ampicillin resistance) gene replaced by the aminoglycoside acetyltransferase (kanamycin resistance) gene from pET9a (Novagen, Madison, WI). Both plasmids direct eukaryotic gene expression from a cassette containing the human cytomegalovirus immediate early I (CMV IE) gene promoter/enhancer, CMV IE 5' untranslated (UT) sequence, and intron A. Following these regulatory elements is a cloning polylinker for insertion of polypeptide coding sequences. Following the polylinker in VR1012 is the 3' UT sequence from the bovine growth hormone gene for polyadenylation and transcriptional termination. In VR1055, the transcriptional terminator region includes a polyadenylation and termination signals derived from the rabbit b-globin gene. VR1033 is identical to VR1012, except that it contains a cap-independent translational enhancer from the encephalomyocarditis virus within the cloning polylinker sequence. This sequence allows the production of two different polypeptides from a single expressed mRNA.

Plasmid VR4101 (murine interferon α (mIFNα)) was constructed by cloning the murine interferon α cDNA into the vector VR1012 vector. The

cDNA was obtained by amplifying the coding sequence from the plasmid RSV-"1 (Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res. 13*: 805-823 (1985); Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res. 13*: 825-839 (1985)), which was provided by Dr. Paula Pitha-Rowe of Johns Hopkins University. Plasmid VR4111 was constructed by transferring the coding sequences from VR4101 to the VR1055 cloning vector. The oligonucleotide primers used for polymerase chain reaction (PCR) were 5'-AACTGCAGATGGCTAGGCTCTGTGCT-3' (SEQ ID No. 15) and 5'-GAAG-ATCTTCATTTCTCTTCTC-TCAG-3' (SEQ ID No. 16). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

Plasmid VR4102 (human interferon α (hIFNα)) was constructed by cloning the human interferon α cDNA into the VR1012 vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4112 was constructed by transferring the coding sequence sequences from VR4102 to the VR1055 cloning vector. Genomic DNA was isolated using the QIAamp Blood Kit (Qiagen, Inc.). The oligonucleotide primers used for PCR were 5'-AACTGCAGATGGCCTC-GCCCTTTGCT-3' (SEQ ID No. 17) and 5'-CGGGATCCTTATTCCTTC-CTCCTTAATC-3' (SEQ ID No. 18). Reaction conditions were 30 cycles of 94^{B}C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

Plasmid VR4150 (human interferon ω (hIFNω)) was constructed by cloning the human IFNω cDNA into the VR1012 cloning vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4151 (SEQ ID No. 1) was constructed by transferring the coding sequences from VR4150 to the VR1055 cloning vector. The oligonucleotide primers used for PCR were 5'-GCTCTAGATGGCCCTCCTGTTCCCT-3' (SEQ ID No. 19) and 5'-GCGG-ATCCTCAAGATGAGCCCAGGTC-3' (SEQ ID No. 20). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

Plasmid VR1110 (murine interleukin-2 (mIL-2)) was constructed by cloning modified murine IL-2 cDNA into the VR1012 vector. The 5' UT sequence and the two amino acids of the leader peptide were replaced with the rat insulin II gene 5' UT sequence and coding region of the first six amino acids of the rat preproinsulin leader peptide. The IL-2 cDNA was then cloned into the BamHI site of VR1012.

Plasmid VR1103 (human interleukin-2 (hIL-2)) is identical to VR1110 with the exception that the murine IL-2 cDNA was replaced with the cDNA for human IL-2 (Parker et. al. 1996).

Plasmid VR4001 (murine interleukin-12 (mIL-12)) was constructed by cloning the cDNA's encoding the two murine subunits p35 and p40 into the VR1033 vector. Both cDNA's were obtained by amplifying the coding sequences from plasmids provided by Dr. Thomas Gajewski of The University of Chicago (J. Immun.,154:5637; J. Immun., 156:1095). The oligonucleotides used for PCR of p35 were 5'- CAT GCC ATG GGT CAA TCA CGC TAC CTC CTC TTT TTG G-3' (SEQ ID No. 23) and 5'- GCG GAT CCT CAG GCG GAG CTC AGA TAG CCC-3' (SEQ ID No. 24). The oligonucleotides used for PCR of p40 were 5'- ACG CGT CGA CAT GTG TCC TCA GAA GCT AAC CAT CTC-3' (SEQ ID No. 21) and 5'- GCG GAT CCC TAG GAT CGG ACC CTG CAG GGA ACA C-3' (SEQ ID No. 22). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

Plasmids VR1223 (luciferase) was constructed by cloning cytoplasmic luciferase gene into the VR1012 vector (Hartikka *et al.*, *Hum. Gene Ther.* *7*:1205-1217, 1996). The source of the cytoplasmic luciferase gene found in VR1223 was the plasmid termed pSP-luc+ which was purchased from Promega. An Avr II - Xba I restriction fragment encoding the luciferase cDNA was transferred from pSP-luc+ to VR1012 to make VR1223.

Plasmid VR1412 (β-galactosidase) was constructed by cloning cytoplasmic β-gal gene into the VR1012 vector (Doh *et al.*, *Gene Ther.* *4*:648-663).

Plasmid VR1332 was constructed by inserting a *Sal*I-*Bam*HI fragment encoding chloramphenicol acetyltransferase (CAT) from pBS-CAT (Promega) into *Sal*I/*Bam*HI-cut VR1012 vector (Hartikka *et al.*, *Hum. Gene Ther.* 7:1205-1217 (1996)).

Example 2

Purification of pDNA

pDNA was transformed into *Escherichia coli* DH10B-competent cells and grown in Terrific Broth (Sambrook, J. *et al.*, in: *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. A.2 (1989)) complemented with 50 μg/ml kanamycin in a 1 Liter shaker flask. Cells were harvested by centrifugation at the end of the exponential growth phase (approximately 16 hr), typically yielding 10 grams of biomass net weight per liter. Covalently closed circular pDNA was isolated by a modified lysis procedure (Horn, N.A. *et al.*, *Human Gene Therapy 6*: 565-573 (1995)) followed by standard double CsCl-ethidium bromide gradient ultracentrifugation with an average yield of approximately 5 mg per liter. Plasmids were ethanol precipitated and resolubilized in saline at 4°C and dialyzed against saline. Endotoxin content was determined by the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Inc., Falmouth, MA). All plasmid preparations were free of detectable RNA. Endotoxin levels were less than 0.6 Endotoxin Units/μg of plasmid DNA. The spectrophotometric A260/A280 ratios were between 1.75 and 2.0.

Example 3

In Vitro Evaluation of Biological Activity of IFNω and IFNα

To assure that the interferon plasmid DNA used in the following examples encoded biologically active interferon, cell proliferation and antiviral assays were performed. All culture medium used in this and following examples was obtained from Life Technologies (Gaithersburg, MD), and all serum was obtained from HyClone (Logan, Utah).

UM449 cells (American Type Culture Collection, Rockville, MD) were plated at a concentration of 2×10^5 cells per well in a 6 well plate and incubated for 24 hours. Plasmid DNA and the lipid, DMRIE/DOPE (1:1) were each diluted to a concentration of 1 mg in 0.5 ml Optimem medium (Life Technologies, Gaithersburg, MD). The lipid DMRIE/DOPE consists of the cationic lipid (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) at a 1:1 mol:mol ratio (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994). DMRIE/DOPE has been shown to be effective for both *in vitro* (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994) and *in vivo* transfection (Stopeck et al., *J. Clin. Oncol.* 15:341-349, 1997 and Rubin et al., *Gene Ther.* 4:419-425, 1997). The lipid mixture and the DNA mixture were then gently mixed. Medium was removed from the cells which were rinsed gently with PBS, followed by addition of the DNA:lipid mixture (1 ml/well). After incubating the cells for 4-5 h at 37°C, one ml of Optimem with 30% fetal calf serum (FCS) was added to each well. Following an overnight incubation at 37°C, one ml of Optimem with 10% FCS was added to each well. Tissue culture supernatants were collected 48 h after the start of the *in vitro* transfection.

a. Antiproliferative activity

To evaluate the antiproliferative and hence, anti-tumor activity of IFNω and IFNα, supernatants from the above described UM449 cells transfected with the interferon or control plasmid DNA were tested in a cell

proliferation assay of murine or human tumor cell lines (ATCC, Rockville,MD) using the Boehringer Mannheim (Indianapolis, IN) Cell Proliferation Kit II (XTT). Murine or human tumor cells were plated in 96 well plates at the desired concentration (cell concentration varied with each cell line evaluated, for example, at a concentration of 5 x 10^3 cells/ml for B16F10 cells and 5 x 10^4 cells/ml for the Cloudman S91 and glioma 261 cells). The plates were incubated at 37°C for 24 hours followed by addition of tissue culture supernatants from UM449 cells *in vitro* transfected with either interferon plasmid DNA or control plasmid DNA. As a positive control for mIFNα plasmid DNA, mIFNα protein (ICN Pharmaceuticals Inc., Costa Mesa, CA) was serially diluted and added to the wells. For the hIFN plasmid DNA, an interferon reference standard (human leukocyte interferon, Sigma Chemical Co., St. Louis, MO) was included in each assay. Following a 24-72 hour incubation, at 37°C, 50 µl of XTT/ECR substrate was added to each well. Plates were incubated for 6-24 hours at 37°C and the optical density (OD) at 490 nm was determined. Increasing amounts of interferon result in inhibition of cell proliferation and a reduction in the OD_{490}. The percent reduction in cell proliferation due to addition of the supernatants was determined by the formula:

$$1 - \frac{OD_{490} \text{ of cells incubated with interferon plasmid DNA supernatants}}{OD_{490} \text{ of cells incubated with control plasmid DNA supernatants}} \times 100$$

As shown in Table 1, both human interferons displayed the characteristic potent anti-proliferative activity against a wide variety of human tumor cell lines, with the most sensitive line being the NIH-OVCAR3 ovarian line and the least sensitive being the SK-OV-3 ovarian line. Also, the supernatants from the mIFNα pDNA (VR4111)-transfected UM449 cells inhibited the proliferation of murine B16F10 melanoma (generous gift from Dr. Suzuki at the University of Texas, Galveston, Texas), murine Cloudman melanoma S91 (American Type Culture Collection, Rockville, MD), and

murine glioma 261 cell lines (Division of Cancer Treatment Tumor Repository, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD) by 40, 42 and 17%, respectively.

Table 1. IFNω (VR4150) and IFNα (VR4102) *in vitro* biological assay: anti-proliferation activity against human tumor cell lines

	% reduction in cell proliferation (compared to control plasmid DNA supernatants)	
Cell line (tissue type)	Interferon ω	Interferon α
NIH-OVCAR3 (ovarian)	60	43
SCC-4 (squamous)	36	36
ACHN (renal)	41	35
A431 (epidermoid)	24	19
SCC-15 (squamous)	29	29
U87MG (glioblastoma)	36	30
A375 (melanoma)	24	21
PC3 (prostate)	20	22
UMUC3 (bladder)	14	6
A549 (lung)	17	15
MCF7 (breast)	18	18
SK-OV-3 (ovarian)	<10	<10

b. Antiviral Activity

An antiviral assay was performed to evaluate the ability of the supernatants from the interferon plasmid DNA-transfected cells to protect murine L929 cells or human A549 cells from infection by murine encephalomyocarditis (EMC) virus (Assay performed at IIT Institute, Chicago, IL). *In vitro* transfections were performed as described above and supernatants were collected from cells transfected with either VR4151 (hIFNT), VR4112 (hIFNα), VR4111 (mIFNα) or VR1055 (control). Antiviral activity of the supernatants was performed by IIT Research Institute (Chicago, IL). The antiviral assay evaluated the degree of

protection of either human A549 or murine L929 cells from infection with murine encephalomyocarditis (EMC) virus. Briefly, 2.5 x 10^4 L929 cells were plated into 96-well plates and incubated for 24 h. Tissue culture supernatants were serially diluted and added to the L929 cells which were incubated for another 24 h. Supernatants were then removed from the wells, the cells were washed and murine EMC virus was added to each well at a multiplicity of infection of 0.04. Assay plates were incubated further for 24 h followed by removal of supernatants, washing of wells, fixation with 5% formalin and staining with 1% crystal violet. Samples with interferon activity protected the cells from virus infection, resulting in darkly stained cell monolayers.

Supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 30,000, 3,000 or 30 Units/ml, respectively, on human A549 cells. When evaluated for antiviral activity on the murine L929 cell line, supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 300, 1000 and 30,000 Units/ml, respectively (Table 2) showing species specificity of the hIFNs for human cells and mIFNs for mouse cells.

Table 2. Antiviral Activity of interferon Plasmid DNA

	Interferon (Units/ml)	
Plasmid	Human cell line	Murine cell line
VR4151 (hIFNω)	30,000	300
VR4112 (hIFNα)	3,000	1,000
VR4111 (mIFNα)	30	30,000

Example 4

Systemic Interferon Therapy; Intramuscular Administration of Cytokine-Expressing Plasmids

Cell lines and tumor models

Murine B16F10 cells were grown in RPMI-1640 (GibcoBRL) and 5% fetal bovine serum (FBS). Murine Cloudman S91 cells were grown in Ham's F-10 medium with 25 mM Hepes, 0.1 mM, non-essential amino acids, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 2.5% FBS and 12.5% horse serum. Human melanoma UM449 cells were grown in RPMI 1640 with 10% FBS.

Murine glioma 261 tumor fragments and M5076 reticulum cell sarcoma cells were obtained from the Division of Cancer Treatment Tumor Repository (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). The glioma 261 tumor fragments ($2mm^3$) were initially implanted into the inguinal region of C57BL/6 mice using a 13 g trocar (Popper Sons, Inc., New Hyde Park, NY). Tumors which grew in the mice were used to establish a tumorigenic cell line. Minced tumor fragments were placed in Iscove's tissue culture medium with 10% FBS. Glioma 261 tumor cells began to attach to the flasks after several days, and the cells were propagated using standard tissue culture techniques. The M5076 cells were grown as ascites in C57BL/6 mice and frozen in liquid nitrogen. Human A431 cells were obtained from the American Type Culture Collection and were grown in DMEM and 10% FBS.

C57BL/6, DBA/2, nude (*nu/nu*) and beige-nude *(bg/nu/xid)* female mice between the ages of 6-8 weeks were obtained from Harlan Sprague Dawley (San Diego, CA). All animal experiments in this and the following examples were conducted in accordance with Vical's Institutional Animal Care and Use Committee as well as the standards set forth in the National Research Council guidelines concerning animal care and use.

To establish subcutaneous B16F10 melanoma tumors, C57BL/6, nude or nude-beige mice were injected subcutaneously on the flank with 10^4

B16F10 cells. The Cloudman melanoma model was established by subcutaneous injection of 10^5 Cloudman S91 cells on the flank of DBA/2 mice and the glioma 261 model was established by subcutaneous injection of 5 x 10^4 glioma 261 cells on the flank of C57BL/6 mice. To establish human epidermoid carcinomas, nude mice were injected subcutaneously on the flank with 5 x 10^3 A431 cells.

To establish intradermal M5076 tumors and liver metastases thereof, C57BL/6 mice were injected intradermally with 10^5 M5076 reticulum cell sarcoma cells. In this model, primary intradermal tumors spontaneously metastasize to the liver. On day 29 after tumor cell injection, mice were sacrificed, livers removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), and the liver nodules were counted.

To establish lung metastases of B16F10 melanoma, C57BL/6 mice were injected intravenously with 2 x 10^4 B16F10 cells. On day 25 after tumor cell injection, the mice were sacrificed, lungs removed and fixed in 10% buffered formalin, and the lung nodules were counted.

To monitor the primary tumor growth, tumor sizes were determined 2-3 times per week by measuring with calipers (l x w x h), and tumor volumes were determined using the formula: tumor volume (mm^3) = 0.52 (l x w x h).

Tumor volume was analyzed using the Mann-Whitney U non-parametric Statistical Test to identify groups having significantly different mean weights. Mouse survival was analyzed using a Kaplan-Meier survival plot followed by a Logrank (Mantel-Cox) test to identify significant differences in survival between groups. Differences were considered statistically significant when the p value was less than or equal to 0.05.

Intramuscular injections

Fifty to 100 μg of plasmid DNA in 50 μl of saline was injected into the rectus femoris muscle of each hind leg for a total DNA dose of 100 to 200 mg. The muscle injections were performed using a 300 ml sterile tuberculin syringe fitted with a 28G ½ needle (Becton Dickenson) and a plastic collar cut

from a 200 ml micropipette tip. The collar length was adjusted to limit the needle from penetrating further than 2 mm into the rectus femoris muscle.

Serum levels of interferon following intramuscular injection of interferon plasmid DNA

Serum samples from C57BL/6 mice injected intramuscularly with either VR4111 (mIFNα plasmid) or VR1055 (control plasmid DNA) were analyzed in a mIFNα ELISA (n=10). For the ELISA, 96-well plates (Immulon 4HBX high binding plates from Dynex Technologies, Chantilly, VA) were coated with rat anti-mouse IFNα monoclonal antibody (mAb) from Caltag Laboratories (Burlingame, CA) at a concentration of 5 μg/ml in 100 mM sodium carbonate buffer, pH 9.5 (50 μl per well). Plates were incubated with the coating mAb for 16 hours at 4°C. The plates were then washed 3 times with a wash buffer (phosphate-buffered saline (PBS)), pH 7.2 and 0.05% Tween-20 (Sigma, St. Louis, MO)). The plates were blocked in PBS containing 3% bovine serum albumin (BSA, Sigma) and 0.05% Tween-20 (400μl per well) and incubated for 24 hours at 4EC followed by washing three times with wash buffer.

Serum samples (10 μl) from mice injected intramuscularly with VR4111 (mIFNα) were mixed with 40 μl of assay buffer (PBS, 1% BSA, 0.05% Tween-20) and the mixture was added to each assay well. The positive control was mIFNα polypeptide (Biosource International, Camarillo, CA), which was serially diluted in assay buffer and 50 μl was added to the positive control wells. The negative control was serum from mice injected intramuscularly with VR1055. After adding the test samples and controls, the plates were incubated for 16 hours at 4°C. The plates were then washed 6 times with wash buffer, followed by addition of a sheep anti-mouse IFNα polyclonal antibody (pAb) (Biosource International, Camarillo, CA). The PAb was added at a 1:500 dilution in assay buffer (50 μl per well) and the plates were incubated for 5 hours at room temperature.

Following incubation with the pAb, the plates were washed six times with wash buffer followed by addition of anti-sheep IgG conjugated with peroxidase (Sigma) at a 1:5000 dilution in assay buffer (50 µl per well) and incubated for 1 hour at room temperature. The plates were washed 6 times with wash buffer and 200 µl of 3,3',5,5'-tetramethylbenzidine liquid substrate (TMB) (Sigma Chemical Co.) was added per well. Plates were incubated at room temperature for 30 minutes, followed by determination of the optical density of the wells at 650 nm. A standard curve was generated by plotting the ng/ml of mIFNα polypeptide versus the optical density at 650 nm. The concentration of mIFNα in the test serum samples was determined from the linear portion of the mIFNα standard curve. The sensitivity of the mIFNα ELISA was 50 ng/ml.

C57BL/6 mice injected intramuscularly with VR4111 had detectable serum levels of mIFNα after 5 intramuscular injections of 100 µg VR4111 (twice a week for two weeks, followed by one injection the next week). The average serum level of mIFNα after 5 intramuscular injections of VR4111 was 1465 ng/ml (average of 16 mice). At the time of this study, no commercial mIFNα ELISA kit had been developed. Since sensitivity of the in-house mIFNα ELISA is 50 ng/ml, lower serum levels of mIFNα could exist in the mice at earlier timepoints, but we were unable to detect this in our ELISA.

To determine the serum levels of hIFNω, C57BL/6 or nude mice received a single intramuscular injection of 100 µg of VR4151 (hIFNω plasmid DNA) or VR1055 (control plasmid DNA) (50 µg per leg bilaterally) in the rectus femoris. Serum samples were collected daily over a two week period and analyzed in the hIFNω ELISA kit (Alexis, San Diego, CA) which was sensitive to 2 pg/ml. Serum samples were collected from 4-5 mice per day. In the C57BL/6 mice, measurable serum levels of hIFNω were detected as early as one day after injection (69 pg/ml) (Figure 2A). In these mice, peak serum levels were found six days after injection (254 pg/ml) and expression continued out to day 14 (50 pg/ml), the final timepoint of the study.

In nude mice, serum levels of IFNω were found as early as one day after injection (133 pg/ml). Peak serum levels were found on day 7 (648 pg/ml) and expression continued out to day 14 (134 pg/ml), the final time point of the study (FIG. 2B). Thus, interferon could be detected in the serum after a single intramuscular injection of an interferon-encoding plasmid DNA.

Systemic interferon treatment inhibits primary tumor growth

As shown in FIGs. 3-5 and FIG. 7A, mice bearing different tumors were found to significantly benefit from intramuscular injection of different cytokines. To test the efficacy of IFNα plasmid, C57BL/6 mice bearing subcutaneous B16F10 melanoma, subcutaneous glioma 261, or intradermal M5076 tumors, or DBA/2 mice bearing subcutaneous Cloudman melanoma were injected with 100 μg either of VR4111 (mIFNα) or VR1055 (control), twice per week for three weeks, beginning on day 4 after tumor cell injection (n = 8-10 mice per group). In all three subcutaneous tumor models, the mice treated intramuscularly with VR4111 had a significant reduction in tumor volume ($p<0.05$) (FIGs. 3A, 3C, and 3E), and a significant enhancement of survival ($p<0.02$) compared to the mice that received the control plasmid (FIGs. 3B, 3D, and 3F). In the intradermal tumor model, mice treated with intramuscular VR4111 had a significant reduction in primary tumor volume ($p<0.001$) compared to the mice that received the control plasmid (FIG. 7A).

To compare the efficacy of IL-2, IL-12 to IFNα plasmids, C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected with 100 μg of VR4111 (mIFNα), VR4001 (mIL-12), VR1110 (mIL-2), or VR1012 (control) (n = 15-16 mice per group) twice per week for three weeks. Mice receiving intramuscular injections of VR4111 had a significant reduction in tumor growth ($p < 0.0002$) (FIG. 4A) by day 17 as well as a significant increase in survival ($p = 0.00001$) (FIG. 4B). By day 28 of the study, 100% of the VR4111-treated mice were still alive, compared to only 20% of the VR1012-treated mice. Mice treated with VR1110 had a modest reduction in tumor

growth by day 17 ($p < 0.02$) (FIG. 4A) but did not have an increase in survival compared to the VR1012-treated mice (FIG. 4B). Mice treated with VR4001 also had a modest reduction in tumor growth by day 17 ($p < 0.03$) (FIG. 4A) as well as a significant increase in survival ($p = 0.02$) (FIG. 4B). By day 28, 55% of the mice treated with VR4001 were alive, compared to 20% of the VR1012-treated mice.

To test the efficacy of IFNω, mice bearing human A431 tumors between 30-80 mm^3 were injected intramuscularly with 200 μg of either VR4151 (hIFNω) or VR1055 (control) twice per week for three weeks (n=15). Mice bearing subcutaneous A431 tumors and injected intramuscularly with VR4151 had a significant reduction in tumor volume ($p < 0.05$) (FIG. 5A) and a significant increase in survival ($p < 0.05$), compared to the mice that received the control plasmid (FIG. 5B).

Systemic mIFNα plasmid DNA treatment inhibits the growth of tumor metastases

As shown in FIG. 6 and FIG. 7, mice bearing different tumor metastases were found to significantly benefit from intramuscular injection of IFNα. C57BL/6 mice bearing lung metastases of B16F10 melanoma were injected intramuscularly with 100 μg of either VR4111 (mIFNα) or VR1055 (control) twice per week for three weeks, beginning on day 4 after tumor cell injection (n=10). On day 25 after tumor cell injection, the mice were sacrificed, lungs removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) followed by counting of lung nodules.

While 70% of the control plasmid-treated mice had lung nodules that were too numerous to count, 80% of the mice treated with mIFNα plasmid DNA had 10 or fewer nodules (FIG. 6). TNTC denotes lungs with nodules that were too numerous to count.

In the liver metastases model, C57BL/6 mice bearing intradermal M5076 murine reticulum sarcoma were injected intramuscularly with 100 μg of either VR4111 or VR1055 twice per week for three weeks, beginning on

day 4 after tumor cell injection (n=10-13 mice per group). On day 29 after tumor cell injection, the mice were sacrificed, livers removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) followed by counting of liver nodules.

While the control plasmid-treated mice had a mean of 190 hepatic tumor nodules or had nodules that were too numerous to count, mIFNα plasmid DNA-treated mice had a mean of 35 hepatic tumor nodules (FIG. 7B).

These results demonstrate that intramuscular injection of mIFNα plasmid DNA can effectively inhibit the growth of both primary and metastatic lesions. Thus, for patients with metastatic disease, intramuscular administration of therapeutic plasmid DNAs would be advantageous for the treatment of undiagnosed or inaccessible metastatic lesions.

Regimen optimization of mIFNα therapy in the B16F10 melanoma model

A regimen optimization study was conducted to evaluate the antitumor efficacy of fewer injections and/or a lower dose of VR4111(mIFNα) in the subcutaneous B16F10 melanoma model. C57BL/6 mice were injected with either 100 or 50 μg of VR4111 or VR1055 over a 6 week period (n=10). Mice received intramuscular injections either twice per week, once per week or once every other week. All intramuscular injections began four days after the initial subcutaneous B16F10 tumor cell injection. Mice which received intramuscular injections of 100 μg of VR4111 at any of the time courses had a significant reduction in tumor volume ($p \leq 0.005$) and a significant increase in survival ($p=0.007$) (FIGs. 8A and 8B). The mice which received the 100 μg dose of VR4111 once every other week for 6 weeks had a total of only three intramuscular injections with significant antitumor efficacy. In contrast, mice receiving the 50 μg dose of VR4111 revealed a dose response based on the frequency of injection. While mice injected with 50 μg of VR4111 once or twice per week had a significant reduction in tumor volume ($p \leq 0.03$), and a significant increase in survival ($p=0.002$), mice injected only once every other

week did not have a significant antitumor response for either tumor growth or survival (FIGs. 8C and 8D).

Mechanism of mIFNα Antitumor Effect

To investigate the role of natural killer (NK) and T cells in mediating the antitumor effect of systemically delivered mIFNα, VR4111 or VR1055 was administered intramuscularly to nude mice (which are T cell deficient) and to beige-nude mice (which are NK and T cell deficient) bearing subcutaneous B16F10 melanoma tumors. Beginning on day 4 after injection of 10^4 B16F10 cells, 50 μg of plasmid DNA in 50 μl of saline was injected into the rectus femoris muscle of each hind leg for a total DNA dose of 100 μg twice per week for three weeks (n=15 mice per group).

There was neither a significant reduction in tumor volume nor enhancement of survival in the nude mice (FIGs. 9A and 9B) or beige-nude mice (FIGs. 9C and 9D). These results suggest that T cells may be required for the mIFNα antitumor response. NK cells appeared not to be required for the antitumor effect since nude mice (NK^+) did not have a greater antitumor response compared to the beige-nude mice (NK^-).

To further explore the role of T cells in the antitumor effect of mIFNα DNA therapy, C57BL/6 mice bearing subcutaneous Bl6F10 tumors were injected with depleting doses of monoclonal antibodies (mAbs) specific for either $CD4^+$ or $CD8^+$ T cells. For depletion of T cell subsets, anti-CD4 (clone GK1.5, rat IgG) and anti-CD8 (clone 2.43, rat IgG) hybridomas (American Type Culture Collection, Rockville, MD) were used to generate the corresponding mAb. The anti-CD8 hybridoma was grown as ascites in nude mice and the mAbs were purified from ascites using ion exchange chromatography (Harlan Bioproducts for Science, San Diego, CA). The anti-CD4 hybridoma was grown *in vitro* with Dulbecco's Modified Eagle Medium, 10% fetal bovine serum and low IgG. The anti-CD4 mAb was purified from tissue culture supernatant by ammonium sulfate precipitation to 30%. The

protein pellet was resolubilized and extensively dialyzed in Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (Zymed Laboratories Inc., San Francisco, CA).

Beginning on day 4 after subcutaneous injection with 10^4 B16F10 cells, mice were injected intramuscularly with 100 μg of either VR4111 or VR1055 twice per week for three weeks. For depletion of $CD4^+$ and $CD8^+$ T cells, mice were injected intraperitoneally with 500 μg of either the anti-CD4 mAb (clone GK1.5, rat IgG) or anti-CD8 mAb (clone 2,43, rat IgG) one day prior to each intramuscular DNA injection (n = 10 mice per group). Control tumor-bearing mice were injected intraperitoneally with 500 μg of normal rat IgG (Simga Chemical Co., St. Louis, MO) (n=10). To assure complete depletion, sentinel mice were injected according to the same regimen, and once per week, spleens were collected, dissociated and assessed for the presence of $CD4^+$ and $CD8^+$ T cells. Spleen cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs (Pharmingen, San Diego, CA) and analyzed by flow cytometry (Cytometry Research Services, San Diego, CA). The depletion of $CD4^+$ and $CD8^+$ T cells was consistently greater than 98%, as determined by cytometry.

The mIFNα DNA therapy significantly reduced tumor growth ($p \leq 0.002$) and enhanced survival ($p \leq 0.008$) of both normal mice and mice depleted of $CD4^+$ T cells, compared to mice injected with control plasmid and treated with normal IgG (FIGs. 10A and 10B). These results suggest that $CD4^+$ T cells are not required for the mIFNα antitumor effect. In contrast, mice depleted of $CD8^+$ T cells and injected with mIFNα DNA displayed tumor volumes and survival profiles that were not significantly different from mice treated with the control plasmid (FIGS. 10A and 10B). This result suggests that $CD8^+$ cells are involved in the mIFNα antitumor response.

Example 5

Local Interferon Therapy: Intratumoral Administration of interferon Plasmids

The anti-tumor activity of IFNω and IFNα was evaluated *in vivo* in nude mice bearing subcutaneous human ovarian (NIH-OVCAR3) or human melanoma (A375) (nude/human/xenograft model), or in C57BL/6 mice bearing murine melanoma (B16F10) tumors following intratumoral administration of DNA complexed with a cationic lipid.

Cell Lines and Tumor Models

Athymic nude *(nu/nu)* and C57/BL/6 mice between the ages of 6-10 weeks were obtained from Harlan Sprague Dawley (San Diego, CA).

Human A375 melanoma cells and human NIH-OVCAR3 ovarian carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco's modified Eagle's medium (GibcoBRL, Gaithersburg, MD) supplemented with 10% FBS. B16F10 cells were a generous gift from Dr. Suzuki at the University of Texas (Galveston,Texas). Cells were grown in RPMI-1640 (GibcoBRL) and 5% fetal bovine serum (FBS).

To establish subcutaneous A375 melanoma tumors and subcutaneous NIH-OVCAR3 ovarian tumors, athymic nude/nude mice (10 mice/group) were injected subcutaneously with 5×10^6 A375 cells and 5×10^7 NIH-OVCAR3 cells respectively. To establish subcubaneous murine B16F10 melanoma tumors, C57BL/6 mice were injected subcutaneously with 10^4 B16F10 cells.

Mice were monitored for tumor growth and survival. Tumor sizes were determined 3 times per week by measuring with calipers (l x w x h) and tumor volumes were determined using the formula: tumor volume (mm^3)= 0.52 (l x w x h). Statistical analysis was done as described in Example 4.

Local Interferon Plasmid DNA Inhibits Tumor Growth

Established subcutaneous A375 human melanoma and NIH-OVCAR3 human ovarian carcinoma tumors in nude mice were transfected *in vivo* by intratumoral administration of pDNA/DMRIE/DOPE (DNA: lipid) complexes (n=10). When tumors became palpable (80-300 mm^3, at day 27 post tumor cell implant for the A375 cells, and at day 41 post tumor cell implant for the NIH-OVCAR3 cells), mice were injected intratumorally with 100 μg of VR4112 (hIFN"), VR4151 (hIFNT), VR1055 (control) or VR1012 (control) complexed with DMRIE/DOPE (1:1 DNA:DMRIE mass ratio). Tumor bearing animals were treated intratumorally with DNA: lipid for 6 consecutive days followed by 5 treatments every other day for a total of 11 treatments (A375 melanoma model), or every other day for a total of 11 treatments (NIH-OVCAR3 ovarian cancer model).

As shown in FIG. 11A, in the A375 model of melanoma, the direct intratumoral injection of VR4151: lipid complex for 6 consecutive days, followed by 5 additional injections every other day (100 μg plasmid DNA/injection, total of 11 injections), resulted in a statistically significant slowing of tumor growth, as compared to the control ($p < 0.03$, days 40-44).

As shown in FIG. 2B, in the NIH-OVCAR3 model of ovarian cancer, the direct intratumoral injection of VR4151: lipid complex every other day for a total of 11 injections (100 μg plasmid DNA/injection), resulted in sustained and statistically significant reduction in tumor growth as compared to the control plasmid control ($p< 0.001$ - 0.05, Days 45-65). A similar treatment regimen with VR4112: lipid was found to have a moderate effect on tumor growth which only reached statistical significance vs. the control plasmid at two time-points during the study ($p < 0.05$, days 53 and 57).

As shown in FIG. 12A, in the B16F10 melanoma model, direct intratumoral injection of mIFNα resulted in decrease in tumor volume. Once palpable tumors were established (80-300mm^3) on day 12 after tumor cell injection in C57BL/6 mice bearing subcutaneous B16F10 melanoma tumors, mice were injected intratumorally with 100 μg of either VR4101 (mIFNα) or

VR1012 (control) complexed with the cationic lipid, DMRIE/DOPE at a DNA/DMRIE mass ratio of 1:1 in a 100 µl volume. Mice received six consecutive intratumoral injections of either VR4101 or VR1012 (n=10 mice per group). As shown in FIG. 12A, although not statistically significant, intratumoral injection of VR4101 resulted in a 54% reduction in tumor volume by day 19 of the study. As shown in FIG. 12B, a significant increase in survival ($p = 0.02$) was found for the VR4101-treated mice, compared to the mice that received VR1012.

Example 6

Local Cytokine Therapy: Intraperitoneal Administration of Cytokine-expressing Plasmids

The goal of this example is to show that the present invention provides an effective method of treating malignant tumors of murine ovarian carcinoma via intraperitoneal (i.p.) injection of cytokine-expressing plasmid DNA. Since late-stage ovarian carcinoma is usually limited to the peritoneal cavity, it was envisioned that continuous secretion of a cytokine in this cavity would produce beneficial anti-tumor immune response. In particular, the present example clearly shows that the ovarian cancer therapy by intraperitoneal injection of a cytokine-expressing plasmid DNA:lipid complexes (1) results in sustained levels of the cytokine in the ascites, avoiding the need for frequent injections of the protein (in contrast to intraperitoneal injection of recombinant cytokine wherein the cytokine level declines shortly after injection), (2) targets tumor ascites, rather than peritoneal tissues (suggesting that systemic cytokine side effects should be reduced using this method), (3) inhibits tumor growth and enhances survival, and (4) can be combined with debulking of tumor ascites to enhance the antitumor effect.

Cell Lines and Tumor Models

As a model for human ovarian cancer, the murine ovarian teratocarcinoma (MOT) model in C3H/HeN mice was used. MOT exhibits many of the characteristics of late-stage human ovarian cancer including peritoneal spread, production of tumor ascites and tumor cell blockage of lymphatics (Ozols et al., 1979 and Fekete et al., 1952).

Murine ovarian teratocarcinoma (MOT) cells were obtained from Dr. Robert Knapp and Dr. Robert C. Bast at the Dana-Farber Cancer Center (Boston, MA). The MOT cells (10^5) were grown by serial intraperitoneal (i.p) transplantation in C3H/HeN mice and a stock of the cells was frozen in liquid nitrogen.

CTLL-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville,MD) and were grown in RPMI 1640 with glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin (Life Technologies, Gaithersburg, MD), 10% fetal bovine serum (HyClone, Logan, Utah) and 10 U/ml murine IL-2 (Boehringer Mannheim, Indianapolis, IN).

C3H/HeN and nude (*nu/nu*) female mice between the ages of 6-10 weeks were obtained from Harlan Sprague Dawley (San Diego, CA). All animal experiments were conducted in accordance with Vical's Institutional Animal Care and Use Committee as well as the standards set forth in the National Research Council guidelines concerning animal care and use.

To establish i.p. MOT tumors, C3H/HeN mice were injected i.p. with 10^5 MOT cells in 100 ul of medium. In the MOT tumor model, tumor growth is typically monitored by weighing the mice which reflects the increase in volume of tumor ascites (Berek et al., *Cancer Res.*, 44:1871-1875, 1984). The nude mouse study were performed in the same manner as the studies in C3H/HeN mice with injection i.p. of 10^5 MOT cells and monitoring the weight of the mice. Statistical analysis on mouse weight and survival was done as described in Example 4.

Preparation of plasmid DNA:lipid complexes and intraperitoneal injection

To yield a pDNA:DMRIE mass ratio of 1:1, 100 μg of VR1110 (mIL-2) was diluted in 500 ul 0.9% saline (Radix Labs, Eau Claire, WI), DMRIE/DOPE lipid (100 μg DMRIE) was diluted in 500 ul of 0.9% saline in a separate vial, and the pDNA and cationic lipid were combined and vortexed for 5 seconds. To yield a pDNA:DMRIE mass ratio of 5:1, 500 μg of mIL-2 pDNA was diluted in 500 ul 0.9% saline (Radix Labs, Eau Claire, WI), DMRIE/DOPE lipid (100 μg DMRIE) was diluted in 500 ul of 0.9% saline in a separate vial, and the pDNA and cationic lipid were combined and vortexed for 5 seconds.

The 1 ml pDNA:DMRIE/DOPE (DNA: lipid) complex was injected i.p. into mice bearing i.p. MOT tumors on various days after tumor cell implant. Control MOT tumor-bearing mice received i.p. injections of VR1012 (control): lipid at the same ratio (1:1 pDNA:DMRIE, 100 μg pDNA) and were injected i.p. on the same days as the cytokine-expressing or reporter gene treatment groups.

Intraperitoneal injection of plasmid DNA:lipid results in targeted expression in tumor ascites

The pDNA:lipid therapy was evaluated for the ability to target malignant cells within a cavity. C3H/HeN mice were injected i.p. with 10^5 MOT cells followed by i.p. injection of 100 μg of VR1223 (luciferase): lipid (1:1 pDNA:DMRIE mass ratio) on days 5 and 6 after tumor cell implant. Control MOT tumor-bearing mice were injected i.p. with 100 μg of either VR1012: lipid or VR1223 without lipid on days 5 and 6 after MOT tumor cell injection. An additional group of control mice did not receive MOT tumor cells and were injected i.p. with VR1223: lipid on the same days as the other treatment groups (n=3). Three days later the mice were euthanized and tumor ascites and tissues (liver, kidney, spleen, diaphragm, intestine and ovary) were collected. Luciferase was extracted from the tissues by freeze-thawing and

grinding of the samples in cell lysis reagent (Promega, Madison, WI) as previously described (Hartikka et al., *Hum. Gene Ther.*, 7:1205-1217, 1996). The tumor ascites was diluted 1:5 in cell lysis reagent followed by three cycles of freeze-thaw and collection of supernatant from the cell lysate. Samples were read in a microplate luminometer (Dynatech, Chantilly, VA) following addition of luciferase substrate (Promega, Madison, WI). The relative light units (RLU) of the samples were determined from a standard curve using purified firefly luciferase (Analytical Luminescence Laboratory, Sparks, MD). The protein concentration of each sample was determined using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL). Luciferase levels were expressed as RLU per mg of protein. There may be a decrease in IL-2 pDNA expression from day 1 to day 3 post DNA injection.

On day three, tumor ascites had 900,000 RLU of luciferase/mg, while diaphragm and ovary tissue had only 327 and 16 RLU/mg (FIG. 13). Kidney, liver, spleen and intestinal tissue had no detectable luciferase activity. These results suggest that i.p. injection of pDNA: lipid complexes appears to target the tumor ascites in the peritoneal cavity with limited or negligible transfection of surrounding tissues. Luciferase detected in the diaphragm and ovary tissue was only found in MOT tumor-bearing mice injected with VR1223: lipid. When naive non-tumor bearing mice were injected with the same DNA: lipid complex, no luciferase activity was found in any of the tissues (data not shown). These results suggest that the low levels of luciferase in the diaphragm and ovary in MOT tumor-bearing mice may reflect metastases of tumor cells to these tissues. Tumor-bearing mice injected with luciferase pDNA without cationic lipid had no luciferase activity in either tumor ascites or surrounding tissues, indicating that lipid is required for optimal *in vivo* transfection of ovarian tumor ascites. Mice injected with VR1012: lipid had no detectable luciferase activity in either tumor ascites or tissues.

A follow-up study investigated the specific cell type in the ovarian tumor ascites that was transfected after i.p. injection of a reporter gene pDNA:DMRIE/DOPE complex. On days 5 and 6 after tumor cell implant (10^5

cells), C3H/HeN mice were injected i.p. with 100 μg of VR1412 (β-galactosidase (β-gal)): lipid, VR1012: lipid (1:1 DNA:DMRIE mass ratio), or with VR1412 without cationic lipid (n=3 mice per group). One day later the mice were sacrificed and the tumor ascites was collected. The ascites was spun at 2500 rpm for 2 minutes to pellet the cells, and the supernatant was removed. The tumor cells were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), placed in a cryomold containing OCT embedding medium (VWR, S. Plainfield, NJ), frozen in isopentane and then stored at -70°C. The embedded and frozen samples were then cryostat sectioned (5 um), further fixed (0.5% glutaraldehyde in PBS), washed (PBS), stained with X-gal reagent (1 mg/ml X-gal diluted in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM magnesium chloride), washed again (PBS), and counterstained with hematoxylin and eosin. (The samples were cryostat sectioned and stained by Pathology Associates (Frederick, MD).)

The ascites from mice treated with either VR1012 or VR1412 without lipid had no β-gal activity in the samples. In contrast, the tumor ascites from mice injected with VR1412: lipid had β-gal staining primarily in the tumor cells (data not shown). In a few slides, several macrophages and lymphocytes were also positive for β-gal while neutrophils were negative for β-gal.

Intraperitoneal injection of IL-2 pDNA:lipid results in sustained expression of IL-2 in tumor ascites

A time-course study was done to determine the length of time that IL-2 could be detected after multiple i.p. injections of IL-2 pDNA: lipid or a single i.p. injection of either IL-2 protein or IL-2 pDNA: lipid in mice bearing i.p. ovarian tumor ascites. Beginning on day 5 after tumor cell injection, mice were injected multiple times with VR1110 (mIL-2) : lipid or a single time with VR1110: lipid or IL-2 protein. Mice were sacrificed at various times and ascites and serum were analyzed for IL-2 levels. Ascites was collected from the sacrificed mice, the samples were spun at 14,000 rpm for 2 minuts and the

supernatant was harvested. Blood was collected from the mice on the same day as the ascites collection and the serum was separated from blood cellls by allowing the blood to clot in serum separator tubes (Microtainer, Becton Dickinson, Franklin Lakes, NJ) followed by centrifugation at 14,000 rpm for 10 minutes and collection of the serum supernatant. IL-2 concentration (pg/ml) in the ascites and serum samples was determined using a murine IL-2 ELISA (R & D Systems, Minneapolis, MN). Since the volume of tumor ascites increases over time, the volume of ascites was also determined for each mouse. The total concentration of IL-2 in ascites was determined using the formula: IL-2 pg/ml x ml of ascites = pg IL-2/ total ascites. Serum IL-2 concentrations were reported as pg/ml.

IL-2 in serum and ascites after multiple injections of IL-2 pDNA: lipid.

Beginning on day 5 after tumor cell injection, mice were injected with VR1110: lipid for either 2, 4 or 6 consecutive days or with control VR1012: lipid for 6 consecutive days (100 µl of plasmid DNA complexed with 100 µl DMRIE/DOPE at 1:1 mass ratio in a total volume of 1 ml). An additional group of mice received VR1110 that was not complexed with DMRIE/DOPE. Every two days for up to 17 days after the DNA:lipid injections, 3-4 mice were sacrificed per treatment group and ascites and serum were collected and analyzed using mIL-2 ELISA assay as described above.

Two injections of VR1110: lipid (100 µl DNA per day, 1:1 DNA:cationic lipid mass ratio) into mice bearing i.p. MOT ovarian tumors yielded high levels of IL-2 protein in the tumor ascites. One day after VR1110: lipid i.p. injection, 28,000 pg/ml of IL-2 was measured in the tumor ascites (Table 3). IL-2 expression in the ascites continued for over two weeks after DNA:lipid injection with 750 pg/ml detected 17 days after the last pDNA:lipid injection. Mice injected with either four or six consecutive injections of VR1110: lipid also had high levels of IL-2 in the tumor ascites; however, due to the very high expression levels after more frequent VR1110: lipid injections, IL-2-mediated side effects were noted in the mice which did

not survive beyond day 9 or 13 after DNA injection. In contrast, two consecutive injections of VR1110 plus DMRIE/DOPE did not cause observable IL-2 side effects yet IL-2 expression levels remained high for over 2 weeks. The IL-2 expressed after i.p. DNA:lipid injection of MOT-bearing mice appeared to remain localized in the peritoneal cavity as the IL-2 serum levels after VR1110: lipid i.p. injection were always less than 10% of the levels in the tumor ascites. Injection of VR1110 without lipid yielded very low levels of IL-2 in ascites and serum (0-32 pg/ml). Injection of the control vector, VR1012, resulted in only background levels of IL-2.

Table 3
mIL-2 concentration in ascites (pg/ml)

Days post tumor cell injection

Treatment	7	9	11	13	15	17	19	21	23
VR1110 (mIL-2) without DM/DP, 6 injs.	28	12	0	1	0	1	0		
VR1012 (control) + DM/DP, 6 injs.	0	0	8	0	0	1	43		
VR1110 (mIL-2) + DM/DP, 6 injs.			7196	7716	7824	3895	3200		
VR1110 (mIL-2) + DM/DP, 4 injs.		3524	4187	5968	3050	3984	2392	441	
VR1110 (mIL-2) + DM/DP, 2 injs.	28882	4750	11725	8047	1246	1445	1407	774	753

mIL-2 concentration in serum (pg/ml)

Days post tumor cell injection

Treatment	7	9	11	13	15	17	19	21	23
VR1110 (mIL-2) without DM/DP, 6 injs.	0	4	0	32	0	0	0		
VR1012 (control) + DM/DP, 6 injs.	0	0	0	0	0	0	62		
VR1110 (mIL-2) + DM/DP, 6 injs.			66	85	34	5	4		
VR1110 (mIL-2) + DM/DP, 4 injs.		114	81	40	10	3	2	0	
VR1110 (mIL-2) + DM/DP, 2 injs.	625	418	181	76	6	0	1	0	0

IL-2 in serum and ascites after single injection of pDNA:lipid injection compared to protein injection.

Five days after i.p. injection of 10^5 MOT tumor cells, C3H/HeN mice were injected with 100 μg of either VR1110: lipid or VR1012: lipid (1:1 DNA:DMRIE mass ratio) or with 100 μg of VR1110 without lipid. For the IL-2 protein-treated group, mice were injected with 1 μg recombinant murine IL-2 protein (R & D Systems, Minneapolis, MN). The pDNA: lipid, pDNA alone, or recombinant protein was injected i.p. in a total volume of 1 ml saline per mouse. Five mice from each group were sacrificed beginning at 4 hours and continuing on days 1, 2, 3, 6 and 10 post DNA or protein injection. Ascites and serum were collected and analyzed using mIL-2 ELISA assay as described above.

Mice injected i.p. with IL-2 protein had peak levels of IL-2 in ascites (10 ng) at 4 hours after injection of IL-2 and a 1000-fold reduction in IL-2 one day later (0.009 ng) (FIG. 14A). In contrast, mice injected with IL-2 pDNA: lipid had peak IL-2 levels in ascites 2 days after injection (64 ng) and only a 2.6 fold reduction in IL-2 by 10 days after injection (25 ng) (FIG. 14A). These results indicate that mice receiving IL-2 pDNA: lipid have more sustained levels of IL-2 in the ascites compared to mice receiving IL-2 protein. Tumor-bearing mice injected i.p. with either VR1012: lipid or VR1110 without cationic lipid had no IL-2 in the tumor ascites. In a related study, MOT tumor-bearing mice injected i.p. with 10-fold less VR1110: lipid (10 μg DNA) still had detectable IL-2 in the tumor ascites 11 days after the VR1110: lipid injection (data not shown).

Serum levels of IL-2 after either i.p. protein or DNA injection reflected a similar pattern as that found in tumor ascites; however, the serum IL-2 levels were markedly reduced compared to the ascites IL-2 levels. Four hours after protein injection, IL-2 in the serum was 2.4 ng/ml and negligible by one day after protein injection. Serum levels of IL-2 one day after VR1110: lipid injection was 1 ng/ml and undetectable by 6 days after the injection (FIG.

14B). These results suggest that the majority of the IL-2 after either IL-2 protein or pDNA: lipid delivery remains in the peritoneal cavity.

Intraperitoneal injection of IL-2 plasmid DNA:lipid inhibits tumor growth and enhances survival

Six consecutive-day treatments. The plasmid DNA:lipid therapy was evaluated for the ability to reduce tumor growth and to increase survival of mice with MOT tumors. On day 5 after MOT tumor cell injection, mice were injected i.p. with 100 μg of either VR1110 or VR1012, both complexed with DMRIE/DOPE. The plasmid DNA was complexed at either a 5:1 or 1:1 DNA:DMRIE mass ratio. An additional treatment group received VR1110 that was not complexed with lipid. A total volume of 1 ml DNA:lipid or DNA alone in physiological saline was injected i.p. The DNA treatments occurred over 6 consecutive days, beginning on day 5 (days 5-10). MOT tumor growth was measured by weighing the mice. All treatment groups consisted of 10 mice per group.

The high IL-2 expression level in ascites was accompanied by significant antitumor effects. Mice treated with VR1110: lipid on days 5-10 after tumor cell injection had a significant reduction in MOT tumor growth compared to the mice treated with the VR1012: lipid ($p=0.01$) (FIG. 15A). A significant enhancement in survival was also found for mice injected i.p. with VR1110: lipid ($p=0.05$) (FIG. 15B).

Complexing the pDNA with a cationic lipid seemed necessary for the antitumor effect as treatment of tumor-bearing mice with VR1110 without lipid was not effective (FIGs. 15E and 15F). Furthermore, a 1:1 DNA:cationic lipid mass ratio was found to be more effective at reducing tumor burden and increasing survival than a 5:1 DNA:cationic lipid mass ratio (FIGs. 15C and 15D).

Three alternative-day treatments. C3H/HeN mice bearing i.p. MOT tumor ascites were injected i.p. with VR1110: lipid or with VR1012: lipid (100 μg DNA) on days 5, 8 and 11 after tumor cell implant. By day 14 post

tumor cell injection, mice treated with VR1110: lipid had a significant reduction in mean weight ($p=0.001$) compared to the mice treated with the control pDNA: lipid (FIG. 16A). In addition, a significant increase in survival ($p=0.008$) was found for the VR1110: lipid-treated mice compared to the mice treated with the VR1012: lipid (FIG. 16B). By day 26 post tumor cell injection, none of the mice treated with the VR1012 were still alive, while 50% of the mice treated with VR1110: lipid remained alive. By day 55 post tumor cell injection, 20% of the mice treated with VR1110: lipid appeared to be tumor-free.

Whether the IL-2 pDNA: lipid antitumor effect required T cells was investigated by implanting nude mice with i.p. MOT tumors followed by same VR1110: lipid regimen used in the C3H/HeN tumor-bearing mice (DNA treatment on days 5, 8 and 11 after tumor cell implant). No significant antitumor effect was found for the nude mice treated with VR1110: lipid suggesting that T cells may be required for the antitumor effect (data not shown).

IL-2 plasmid DNA:lipid antitumor effect enhanced by debulking of tumor ascites

Debulking of tumor ascites is commonly performed on human ovarian cancer patients. A similar procedure was performed in the mice bearing MOT tumors and treated with VR1110: lipid. Mice bearing MOT tumors and injected i.p. with 100 ul DNA:lipid on days 5-10 as described above (1:1 DNA:lipid mass ratio), were also debulked of tumor ascites on day 14 after tumor cell injection (4 days after the last DNA:lipid injection). Mice were debulked of 5 ml of tumor ascites by insertion of a 22 G needle attached to a 5 ml syringe and removal of 5 ml of fluid. The mice were anesthetized with methoxyflurane during the debulking procedure. All treatment groups in this experiment consisted of 8-10 mice per group.

Debulking of ovarian tumor ascites in mice previously treated with IL-2 plasmid DNA:lipid further enhanced the efficacy of the treatment resulting

in a significant reduction in tumor growth (p=0.01) and an increase in survival. Forty four percent of the IL-2 plasmid DNA:lipid-treated and debulked mice were alive on day 57 vs. 17% of the plasmid control-treated and debulked mice (FIG. 17). These results show that plasmid-mediated gene therapy in combination with conventional procedures such as debulking of tumor ascites may hold promise for future treatment of human ovarian cancer.

Dose-response of IL-2 pDNA: lipid

A dose-response study was initiated to determine the minimum dose of VR1110: lipid that could still result in a significant antitumor effect. C3H/HeN mice were injected with 25, 50 or 100 μg of IL-2 pDNA: lipid on days 5, 8 and 11 after MOT tumor cell injection. A control group of MOT tumor-bearing mice were injected with 100 μg of VR1012 complexed with lipid. By day 15 post tumor cell injection, mice treated with either the 50 or 100 μg dose of VR1110 complexed with lipid had a significant inhibition of tumor growth (p=0.002) compared to the mice treated with the VR1012: lipid (FIG. 18A). A significant increase in survival (p=0.01) was also found for the mice treated with either the 50 or 100 μg dose of VR1110: lipid (FIG. 18B). On day 25, none of the mice treated with the VR1012: lipid survived, while the mice injected with 50 or 100 μg of VR1110: lipid had 27 and 33% survival, respectively. By day 37, mice treated with 50 or 100 μg of VR1110: lipid had 20 and 27% survival, respectively. Tumor-bearing mice treated with 25 μg of VR1110 complexed with lipid were not significantly different from the control mice for either tumor volume or survival.

Cytokine profile of ovarian tumor ascites

Since i.p. injection of IL-2 pDNA: lipid into mice bearing i.p. MOT tumors resulted in high levels of IL-2 expression in the ascites, it was of interest to determine whether the IL-2 therapy initiated a cytokine cascade in

the tumor ascites. C3H/HeN mice were injected i.p. with 10^5 MOT cells. On days 5, 8 and 11 after tumor cell implant, the mice were injected i.p. with either VR1110: lipid or VR1012: lipid (1:1 pDNA:DMRIE mass ratio) or received no treatment after the MOT tumor cell injection. Two days after each injection of pDNA: lipid (days 7, 10 and 13 after tumor cell implant), 5 mice per group were sacrificed and the tumor ascites was collected. The total volume of ascites was determined per mouse. The ascites samples were spun at 14,000 rpm for 2 minutes followed by collection of the supernatants. The ascites supernatants were assayed for the concentration of the cytokines: IL-2, IL-4, IL-6, IL-10, IL-12, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) using ELISA (R & D Systems, Minneapolis, MN). The concentration of transforming growth factor beta (TGFβ) in the ascites was assayed using the $TGF\beta_2$ Emax Immunoassay System (Promega, Madison, WI). The amount of cytokine in the tumor ascites was calculated using the formula: cytokine concentration in pg/ml x ml of total ascites = pg of cytokine/total ascites.

As expected, tumor-bearing mice injected i.p. with VR1110: lipid had a marked increase in IL-2 levels with negligible levels in untreated tumor-bearing mice or mice injected with the VR1012: lipid (FIG. 19A). The levels of IFNγ and GM-CSF were also markedly elevated in the mice treated with VR1110: lipid (FIGs. 19B and 19C). The IFNγ and GM-CSF levels in these mice increased on days 10 and 13 after tumor cell injection but not on the day 7 timepoint suggesting that this could be due to IL-2 secretion. Some non-specific increase in IFNγ was also found in the ascites of tumor-bearing mice after injection of the VR1012: lipid;however, by day 13, the levels of IFNγ in the tumor-bearing mice treated with VR1110: lipid were 6-fold higher than in the mice treated with VR1012: lipid suggesting that expression of IL-2 upregulates IFNγ production.

Levels of IL-6, TNFα and IL-10 were increased in both the IL-2 pDNA: lipid group as well as the control pDNA: lipid group suggesting that pDNA: lipid complexes may non-specifically stimulate production of these

particular cytokines in the tumor ascites (FIGs. 19D, 19E, and 19F). No differences were found for IL-4, IL-12 or TGFβ in the ascites from any of the groups and levels of these cytokines were low (0-300 pg/ml for IL-4 and IL-12 and 0-2000 pg/ml for TGFβ, data not shown). For all of the cytokines evaluated, mice treated with control pDNA without lipid, IL-2 pDNA without lipid or with lipid alone had similar cytokine levels as the untreated mice.

Intraperitoneal injection of IFNα pDNA:lipid enhances survival

C3H/HeN mice were injected i.p. with 10^5 MOT cells to establish ovarian i.p. tumors. The mice then received i.p. injections of 100 μg of VR4111 (mIFNα) or VR1012 (control) complexed with DMRIE:DOPE cationic lipid at a 1:1 DNA:DMRIE mass ratio in a total volume of 1 ml saline. The mice received the i.p. injections of pDNA: lipid on days 5, 8 and 11 after tumor cell injection. The mice were weighed 3-6 times per week. Fifteen mice were included in each treatment group.

Mice bearing i.p. MOT tumors and treated with i.p. VR4111: lipid had a significant increase in survival ($p<0.006$) compared to the mice receiving the control plasmid (FIG. 20B). No significant reduction in tumor volume was found for the VR4111: lipid-treated mice (FIG. 20A).

Example 7

Selective Transfection of Malignant Cells in Murine Intraperitoneal Melanoma Tumor Model

The anti-tumor effect of DNA formulations with or without lipids in mouse i.p. melanoma model have been evaluated in the present example.

Cell line and tumor model

B16F10 mouse melanoma cells were grown *in vitro* in DMEM and 10% FCS. Two hundred thousand B16F10 mouse melanoma cells were implanted i.p. in C57BL/6 mice in 1-3 ml saline using a 28 G 1/2 needle and without puncturing internal organs.

Preparation of plasmid DNA:lipid complexes

The plasmid DNA-cationic lipid formulations were prepared just prior to use. Equal volumes of DNA and DMRIE : DOPE (1:1) were mixed by swirling to achieve a target concentration of 0.5 mg DNA/ml, 100 μg DMRIE/ml and 0.12 mg DOPE/ml as previously described (Parker et al, 1996; Saffran et al, 1998). The other cationic lipids were mixed similarly so that the mass ratio of DNA to cationic lipid was 5:1 and the cationic lipid to DOPE molar ratio was 1:1. The formulated material was then vortexed at high speed for 30 seconds and kept at room temperature until dose administration.

CAT assay

Tumor or other tissues were collected, immediately frozen in liquid nitrogen and ground into a powder using a reversible drill as described (Manthorpe,M., Hartikka, J., Vahlsing, H.L. and Sawdey, M. Quantification of plasmid DNA transfection in vivo. In Gene Quantification. F. Ferre, ed. Birkhauser, Boston, Ma., 1998 in press.). The dry frozen powder was thawed and extracted in lysis buffer and high speed supernates assayed for CAT activity using a two-phase partition assay as described (Sankaran, L., Analytical Biochemistry 200: 180-186 (1992)).

Cationic lipids enhances tumor transfection

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with CAT pDNA (VR1332):DMRIE/DOPE. Two days later, tumor tissues were collected, extracted, and assayed for CAT activiy.

As shown in Table 4, DNA alone transfected tumor, but DMRIE:DOPE increased transfection by 78 fold (from 2,326 to 182,052).

Table 4

pgs CAT per gm of tumor tissue collected

(n = 4 to 6 mice as indicated); saline values subtracted

Mouse #	DNA only	DNA:DMRIE	DMRIE, no DNA
1	0	82,600	0
2	307	137,551	0
3	706	220,600	0
4	791	287,458	0
5	5,892		0
6	6,258		
Average	2,326	182,052	0
Std Error	1,306	52,140	0
Fold higher	1	78	0

Selective transfection of tumor cells

Normal animal. Normal, non-tumor bearing BALB/c mice were injected i.p. with 1mg/2ml VR1332, with or without a cationic lipid, and with or without the neutral lipid, DOPE. Two days later, selected i.p. tissues (liver, lung, kidney, spleen, mesentery) were collected, extracted, and assayed for CAT activity.

As shown in Table 5, normal intraperitoneal organs were not at all transfected or transfected very little with a variety of cationic lipids: There was a low level transfection of mesentery tissues, and a moderate transfection of 1 of 5 spleens (the one transfected spleen may have been punctured by the injection needle).

Table 5

Average pgs CAT per gm of tumor tissue (avg., n = 5 mice)
Saline background values have been subtracted

ORGANS	DNA only	+ DMRIE	+ ßAE-DMRIE	+ GAP-DLRIE
Liver	0	0	0	0
Lung	0	0	0	0
Kidney	0	0	0	0
Spleen	0	2,563(0)*	0	0
Mesentery	370	1,549	1,866	1,127

ORGANS	+ DOSPA	+ DMRIE, no DOPE	+ ßAE-DMRIE, no DOPE
Liver	0	0	0
Lung	0	0	0
Kidney	0	0	0
Spleen	0	0	0
Mesentery	600	1,091	3,604

*one mouse with a CAT value; the rest = 0

Tumor-bearing animal compared to normal animal. C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with VR1332 with or without cationic lipid/DOPE. Two days later, selected i.p. tissues (liver, lung, kidney, spleen, mesentery) were collected, extracted, and assayed for CAT activity.

As shown in Table 6, tumor tissues were transfected at high levels with pDNA complexed with cationic lipid/DOPE. Normal i.p. organs were not transfected well compared to i.p. tumor tissues.

Table 6

Average pgs CAT per gm of tumor tissue (n = 5 mice); saline values subtracted

ORGANS	DNA only	DNA + DMRIE:DOPE	DNA + DMRIE:DOPE normal mice no tumors
Tumor	0	200,195	n/a
Liver	0	0	0
Lung	0	0	0
Kidney	0	0	0
Spleen	493	0	0
Mesentery	6,795	nd	2,562
Ovary	nd	nd	0

Dose Response

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with 0.15 or 1.5 mgs of VR1332 with or without cationic lipid/DOPE in 3 ml saline. Two days later, tumor tissues were collected, extracted, and assayed for CAT activity.

As shown in Table 7, a higher dose of DNA transfected tumor tissues better than a lower dose. Also, DMRIE transfected better than the two other cationic lipids tested.

Table 7

Average pgs CAT per gm of tumor tissue (n = 5 mice); saline values subtracted

DNA DOSE	DNA only	+ DMRIE	+ GAP-DMRIE	+ PA-DELO
1.5 mgs	16,592	1,270,466	524,006	581,616
0.15 mgs	1,937	131,089	47,866	99,797

Testing a variety of cationic lipids

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with 1 mg of VR1332 with or without cationic lipid/DOPE in 3 ml saline. Two days later, tumor tissues were collected, extracted, and assayed for CAT activiy.

As shown in Table 8, all cationic lipids tested increased transfection level, and DMRIE was one of three preferred cationic lipids.

Table 8

Average pgs CAT per gm of tumor tissue (n = 5 mice); saline values have been subtracted

CL:DOPE	Run 1	Run 2	Average
None	6,247	not done	6,247
GAP-DDRIE	20,816	7,027	13,922
GMU-DMRIE	9,355	52,888	31,122
HP-DORIE	36,615	26,795	31,705
DOSPA	33,494	57,026	45,260
PA-TELO	63,326	32,681	48,004
GA-LOE-BP	59,729	63,528	61,629
GAP-DMRIE	80,760	63,002	71,881
PA-DELO	73,692	82,265	77,979
GAP-DLRIE	77,553	107,629	92,591
DMRIE	77,128	122,225	99,677
DLRIE	122,999	128,225	125,612
PA-DEMO	155,369	154,884	155,127

In sum, the success of the intra-cavity delivery embodiment of the present invention is also exemplified in the murine melanoma tumor model. Following i.p. injection of a polynucleotide, transfection occurs predominantly in tumor tissues, and normal intraperitoneal organs, such as liver, lung, and kidney are poorly transfected, if at all, with the polynucleotide formulation.

It clear that the inventention may be practiced otherwist than as particular described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are withing the scope of the appended claims.

The entire disclosure of all publications (including patents, patent application, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

What Is Claimed Is:

1. A pharmaceutical composition comprising about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group consisting of:

(a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof,

wherein said polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*;

(b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein said polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and

(c) a polynucleotide that encodes a polypeptide comprising amino acids 86 to 172 in SEQ ID No. 8, wherein said polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and

one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

2. The pharmaceutical composition of claim 1, wherein said polynucleotide is (a).

3. The pharmaceutical composition of claim 1, wherein said polynucleotide is (b).

4. The pharmaceutical composition of claim 1, wherein said polynucleotide is (c).

5. The pharmaceutical composition of claim 2, wherein said polynucleotide encodes a polypeptide comprising amino acids -23 to 172 of SEQ ID No. 8.

6. The pharmaceutical composition of claim 2, wherein said polynucleotide encodes a polypeptide comprising amino acids 1 to 172 of SEQ ID No. 8.

7. The pharmaceutical composition of claim 3, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 15 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

8. The pharmaceutical composition of claim 7, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 10 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

9. The pharmaceutical composition of claim 8, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 5 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

10. The pharmaceutical composition of claim 1, wherein said polynucleotide further encodes one or more additional molecules.

11. The pharmaceutical composition of claim 1, wherein said polynucleotide further encodes one or more additional cytokines.

12. The pharmaceutical composition of claim 4, wherein said polypeptide further comprises amino acids 61 to 85 of SEQ ID No. 8.

13. The pharmaceutical composition of claim 12, wherein said polypeptide further comprises amino acids 41 to 60 of SEQ ID No. 8.

14. The pharmaceutical composition of claim 13, wherein said polypeptide further comprises amino acids 21 to 40 of SEQ ID No. 8.

15. The pharmaceutical composition of claim 1, wherein said cationic compound is one or more cationic lipids.

16. The pharmaceutical composition of claim 15, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide, dipalmitoyl-phophatidylethanolamine 5-carboxy-spennylamide, {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

17. The pharmaceutical composition of claim 15, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

18. The pharmaceutical composition of claim 15, further comprising one or more neutral lipids.

19. The pharmaceutical composition of claim 18, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

20. The pharmaceutical composition of claim 18, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

21. The pharmaceutical composition of claim 18, wherein the mass ratio of said polynucleotide construct to lipid is from about 5:1 to about 1:1.

22. The pharmaceutical composition of claim 1, wherein the polynucleotide encoding the polypeptide is DNA operably linked to a promoter.

23. The pharmaceutical composition of claim 1, wherein the polynucleotide encoding the polypeptide is RNA.

24. The pharmaceutical composition of claim 1, wherein said polynucleotide construct is VR4151 complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4151 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

25. The pharmaceutical composition of claim 1, wherein said polynucleotide comprises a region regulating gene expression.

26. The pharmaceutical composition of claim 25, wherein said region regulating gene expression is cell specific or tissue specific.

27. The pharmaceutical composition of claim 26, wherein said region is tumor cell or tumor tissue specific.

28. A pharmaceutical composition obtained by complexing about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group consisting of:

(a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof,

wherein said polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*;

(b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein said polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and

(c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 of SEQ ID No. 8, and wherein said polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro;*

with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

29. The pharmaceutical composition of claim 28, wherein said polynucleotide is (a).

30. The pharmaceutical composition of claim 28, wherein said polynucleotide is (b).

31. The pharmaceutical composition of claim 28, wherein said polynucleotide is (c).

32. The pharmaceutical composition of claim 29, wherein said polynucleotide encodes a polypeptide comprising amino acids -23 to 172 of SEQ ID No. 8.

33. The pharmaceutical composition of claim 29, wherein said polynucleotide encodes a polypeptide comprising amino acids 1 to 172 of SEQ ID No. 8.

34. The pharmaceutical composition of claim 30, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 15 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

35. The pharmaceutical composition of claim 34, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 10 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

36. The pharmaceutical composition of claim 35, wherein said polynucleotide encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 5 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8.

37. The pharmaceutical composition of claim 28, wherein said polynucleotide further encodes one or more additional molecules.

38. The pharmaceutical composition of claim 28, wherein said polynucleotide further encodes one or more additional cytokines.

39. The pharmaceutical composition of claim 31, wherein said polypeptide further comprises amino acids 61 to 85 of SEQ ID No. 8.

40. The pharmaceutical composition of claim 39, wherein said polypeptide further comprises amino acids 41 to 60 of SEQ ID No. 8.

41. The pharmaceutical composition of claim 40, wherein said polypeptide further comprises amino acids 21 to 40 of SEQ ID No. 8.

42. The pharmaceutical composition of claim 23, wherein said cationic compound is one or more cationic lipids.

43. The pharmaceutical composition of claim 42, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide, dipalmitoyl-phophatidylethanolamine 5-carboxy-spennylamide, {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

44. The pharmaceutical composition of claim 42, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

45. The pharmaceutical composition of claim 42, further comprising one or more neutral lipids.

46. The pharmaceutical composition of claim 45, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

47. The pharmaceutical composition of claim 45, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

48. The pharmaceutical composition of claim 45, wherein the mass ratio of said polynucleotide construct to lipid is from about 5:1 to about 1:1.

49. The pharmaceutical composition of claim 28, wherein the polynucleotide encoding the polypeptide is DNA operably linked to a promoter.

50. The pharmaceutical composition of claim 28, wherein the polynucleotide encoding the polypeptide is RNA.

51. The pharmaceutical composition of claim 28, wherein said polynucleotide construct is VR4151 complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4151 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

52. The pharmaceutical composition of claim 28, wherein said polynucleotide comprises a region regulating gene expression.

53. The pharmaceutical composition of claim 52, wherein said region regulating gene expression is cell specific or tissue specific.

54. The pharmaceutical composition of claim 53, wherein said region is tumor cell or tumor tissue specific.

55. A method of treating cancer or metastasis thereof in a mammal, comprising:

administering into a tissue of said mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active fragment thereof,

such that said polynucleotide is expressed as said cytokine *in vivo*, and

such that said cytokine is present in the blood stream of said mammal in an amount effective to treat said cancer.

56. The method of claim 55, wherein said polynucleotide construct is administered free from ex vivo cells or ex vivo cellular material.

57. The method of claim 55, wherein said cancer is selected from the group consisting of renal cell carcinoma, colorectal carcinoma, lymphoma, Kaposi's sarcoma, melanoma, prostate cancer, ovarian cancer, lung cancer, liver cancer, head and neck cancer, bladder cancer, uterine cancer, bone cancer, leukemia, breast cancer, non-melanoma skin cancer, glioma, solid cutaneous tumor, metastases of any of thereof, and combinations of any of thereof.

58. The method of claim 57, wherein said cancer is one or more metastases of one or more of the cancers of claim 57.

59. The method of claim 57, wherein said cancer is lung or liver metastasis.

60. The method of claim 55, wherein said construct is administered into a tissue selected from the group consisting of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue.

61. The method of claim 60, wherein said tissue is muscle.

62. The method of claim 61, wherein said tissue is skeletal muscle, smooth muscle, or myocardium.

63. The method of claim 55, wherein said construct is administered intravenously.

64. The method of claim 55, wherein said construct is administered intramuscularly.

65. The method of claim 55, wherein said polynucleotide construct comprises a polynucleotide encoding a cytokine selected from the group consisting of IFNω, IFNα, IFNτ, IFNγ, IFNβ, IL-1, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GM-CSF, an active fragment of any of thereof, and a combination of any of thereof.

66. The method of claim 65, wherein said cytokine is an interferon ω.

67. The method of claim 66, wherein said interferon ω is a polypeptide comprising amino acids -23 to 172 in SEQ ID No. 8.

68. The method of claim 66, wherein said interferon ω is a polypeptide comprising amino acids 1 to 172 in SEQ ID No. 8.

69. The method of claim 66, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4).

70. The method of claim 65, wherein said cytokine is an interferon α.

71. The method of claim 70, wherein said interferon α is a polypeptide comprising amino acids -23 to 166 of SEQ ID No. 10.

72. The method of claim 70, wherein said interferon α is a polypeptide comprising amino acids 1 to 166 of SEQ ID No. 10.

73. The method of claim 70, wherein said polynucleotide construct is VR4112 (SEQ ID No. 2).

74. The method of claim 65, wherein said cytokine is an interleukin-2.

75. The method of claim 74, wherein interleukin-2 is a polypeptide comprising amino acids 58 to 105 of SEQ ID No. 14.

76. The method of claim 74, wherein interleukin-2 is a polypeptide comprising amino acids 20 to 126 of SEQ ID No. 14.

77. The method of claim 74, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25).

78. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon ω is administered intramuscularly to treat melanoma or metastasis thereof.

79. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon ω is administered intramuscularly to treat metastasis of melanoma.

80. The method of claim 79, wherein the metastasis of melanoma is lung metastasis.

81. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon ω is administered to treat glioma.

82. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon α is administered intramuscularly to treat melanoma.

83. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon α is administered intramuscularly to treat metastasis of melanoma.

84. The method of claim 83, wherein the metastasis of melanoma is lung metastasis.

85. The method of claim 55, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon α is administered to treat glioma.

86. The method of claim 55 wherein said mammal is a human.

87. The method of claim 55 wherein said construct is free from association with transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

88. The method of claim 55, wherein said construct is administered as a complex of said construct and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

89. The method of claim 88, wherein said one or more cationic compounds are one or more cationic lipids.

90. The method of claim 89, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide, dipalmitoyl-phophatidylethanolamine 5-carboxy-spermylamide, {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

91. The method of claim 89, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

92. The method of claim 89, wherein said complex further comprising one or more neutral lipids.

93. The method of claim 89, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

94. The method of claim 94, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

95. The method of claim 94, wherein the ratio of said polynucleotide construct to lipid is about 5:1 to about 1:1.

96. The method of claim 55, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the ratio of VR4151 to lipid is about 5:1 to 1:1.

97. The method of claim 55, wherein said polynucleotide construct is VR4112 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4112 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

98. The method of claim 55, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR1103 (SEQ ID No. 25) to lipid is from about 5:1 to about 1:1.

99. The method of claim 55, wherein said polynucleotide is DNA operably linked to a promoter.

100. The method of claim 55, wherein said polynucleotide is RNA.

101. The method of claim 55, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional molecule.

102. The method of claim 55, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional cytokines.

103. The method of claim 55, wherein said polynucleotide comprises a region regulating gene expression.

104. The method of claim 103, wherein said region regulating gene expression is cell specific or tissue specific.

105. The method of claim 104, wherein said region is tumor cell or tumor tissue specific.

106. A method of treating cancer, or metastasis thereof, in a mammal, comprising:

the method of claim 55 in combination with one or more additional cancer treatment methods selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, and gene therapy.

107. The method of claim 106, wherein said polynucleotide construct is administered prior to the commencement of said one or more additional cancer treatment methods.

108. The method of claim 106, wherein said polynucleotide construct is administered during the practice of said at one or more additional cancer treatment methods.

109. The method of claim 106, wherein said polynucleotide construct is administered after the end of said one or more additional cancer treatment methods.

110. A method of treating cancer in a mammal, comprising:

administering into a tissue of said mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active fragment thereof, selected from the group consisting of an interferon ω, an interferon α, a combination of an interferon ω and an interferon α, and an active fragment of any of thereof, such that said cytokine is delivered to a tumor in a therapeutically effective amount.

111. The method of claim 110, wherein said polynucleotide construct is administered free from *ex vivo* cells or *ex vivo* cellular material.

112. The method of claim 110, wherein said cancer is selected from the group consisting of solid tumor, cutaneous tumor, renal cell carcinoma, colorectal carcinoma, lymphoma, Kaposi's sarcoma, melanoma, prostate cancer, ovarian cancer, lung cancer, head and neck cancer, bladder cancer, uterine cancer, bone cancer, leukemia, breast cancer, non-melanoma skin cancer, glioma, metastases thereof, and combinations thereof.

113. The method of claim 110, wherein said polynucleotide construct is administered directly into a tissue selected from the group consisting of muscle, skin, breast, prostate, blood, bone marrow, lung, heart, liver, kidney, small intestine, colon, and brain.

114. The method of claim 110, wherein said construct is administered intravenously.

115. The method of claim 114, wherein said construct is targeted to a tumor.

116. The method of claim 114, wherein said construct is targeted to a tumor by a promoter.

117. The method of claim 110, wherein said construct is administered directly into a tumor.

118. The method of claim 117, wherein said tumor is a tumor of tissue selected from the group consisting of skin, breast, prostate, blood, bone marrow, lung, heart, liver, kidney, small intestine, colon, brain, and ovary.

119. The method of claim 110, wherein said construct is administered into cells surrounding a tumor.

120. The method of claim 110, wherein said construct is injected into a cavity within which a tumor is found.

121. The method of claim 110, wherein said construct is injected intraperitoneally.

122. The method of claim 110, wherein said construct is administered into one or both lungs of said mammal.

123. The method of claim 110, wherein said cytokine is an interferon ω.

124. The method of claim 123, wherein interferon ω is a polypeptide comprising amino acids -23 to 172 in SEQ ID No. 8.

125. The method of claim 123, wherein interferon ω is a polypeptide comprising amino acids 1 to 172 in SEQ ID No. 8.

126. The method of claim 123, wherein said polynucleotide construct is VR4151 (SEQ ID No. 44).

127. The method of claim 110, wherein said cytokine is an interferon α.

128. The method of claim 127, wherein interferon α is a polypeptide comprising amino acids -23 to 166 of SEQ ID No. 10.

129. The method of claim 127, wherein interferon α is a polypeptide comprising amino acids 1 to 166 of SEQ ID No. 10.

130. The method of claim 127, wherein said polynucleotide construct is VR4112 (SEQ ID No. 2).

131. The method of claim 110, wherein a polynucleotide construct comprising a DNA molecule encoding an interferon ω is administered directly into a tumor to treat melanoma.

132. The method of claim 110, wherein said mammal is a human.

133. The method of claim 110, wherein said construct is free from association with transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

134. The method of claim 110, wherein said construct is administered as a complex of said construct and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

135. The method of claim 134, wherein said one or more cationic compounds are one or more cationic lipids.

136. The method of claim 135, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide , dipalmitoyl-phophatidylethanolamine 5-carboxy-spermylamide, {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

137. The method of claim 135, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

138. The method of claim 135, wherein said complex further comprising one or more neutral lipids.

139. The method of claim 135, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

140. The method of claim 138, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-

bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

141. The method of claim 140, wherein the ratio of said polynucleotide construct to lipid is about 5:1 to about 1:1.

142. The method of claim 110, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the ratio of VR4151 to lipid is about 5:1 to 1:1.

143. The method of claim 110, wherein said polynucleotide construct is VR4112 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4112 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

144. The method of claim 110, wherein said polynucleotide is DNA operably linked to a promoter.

145. The method of claim 110, wherein said polynucleotide is RNA.

146. The method of claim 110, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional molecule.

147. The method of claim 110, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional cytokines.

148. The method of claim 110, wherein said polynucleotide comprises a region regulating gene expression.

149. The method of claim 148, wherein said region regulating gene expression is cell specific or tissue specific.

150. The method of claim 149, wherein said region is tumor cell or tumor tissue specific.

151. A method of treating cancer, or metastasis thereof, in a mammal, comprising:

the method of claim 110 in combination with one or more additional cancer treatment methods selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, and gene therapy.

152. The method of claim 151, wherein said polynucleotide construct is administered prior to the commencement of said one or more additional cancer treatment methods.

153. The method of claim 151, wherein said polynucleotide construct is administered during the practice of said at one or more additional cancer treatment methods.

154. The method of claim 151, wherein said polynucleotide construct is administered after the end of said one or more additional cancer treatment methods.

155. A method of treating cancer in a mammal, comprising:

administering into a body cavity of said mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active fragment thereof, such that said cytokine is delivered to a tumor in a therapeutically effective amount.

156. The method of 155, wherein said cytokine is selected from the group consisting of IFNω, IFNα, IFNτ, IFNγ, IFNβ, IL-1, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GM-CSF, an active fragment of any of thereof, and a combination of any of thereof.

157. The method of claim 156, wherein said cytokine is an interferon ω.

158. The method of claim 157, wherein said interferon ω is a polypeptide comprising amino acids -23 to 172 in SEQ ID No. 8.

159. The method of claim 157, wherein said interferon ω is a polypeptide comprising amino acids 1 to 172 in SEQ ID No. 8.

160. The method of claim 157, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4).

161. The method of claim 156, wherein said cytokine is an interferon α.

162. The method of claim 161, wherein said interferon α is a polypeptide comprising amino acids -23 to 166 of SEQ ID No. 10.

163. The method of claim 161, wherein said interferon α is a polypeptide comprising amino acids 1 to 166 of SEQ ID No. 10.

164. The method of claim 162, wherein said polynucleotide construct is VR4112 (SEQ ID No. 2).

165. The method of claim 156, wherein said cytokine is an interleukin-2.

166. The method of claim 165, wherein interleukin-2 is a polypeptide comprising amino acids 58 to 105 of SEQ ID No. 14.

167. The method of claim 165, wherein interleukin-2 is a polypeptide comprising amino acids 20 to 126 of SEQ ID No. 14.

168. The method of claim 165, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25).

169. The method of claim 155, wherein said polynucleotide construct is administered free from *ex vivo* cells or *ex vivo* cellular material.

170. The method of 155, wherein said tumor disseminates in a body cavity.

171. The method of 170, wherein the body cavity is selected from the group consisting of peritoneal cavity, thoracic cavity, plural cavity, rectal cavity, stomach cavity, and urinary bladder vesicle.

172. The method of 171, wherein the body cavity is peritoneal cavity.

173. The method of claim 155, wherein said cancer is selected from the group consisting of renal cell carcinoma, colorectal carcinoma, lymphoma, melanoma, prostate cancer, ovarian cancer, lung cancer, stomach cancer, glandular lymphoma, bladder cancer, uterine cancer, mesenteric cancer, pancreatic cancer, gastric cancer, metastases thereof, and combinations thereof.

174. The method of claim 155, wherein said construct is targeted to a tumor.

175. The method of claim 155, wherein said construct is injected intraperitoneally.

176. The method of claim 155, wherein said mammal is a human.

177. The method of claim 155, wherein said construct is free from association with transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

178. The method of claim 155, wherein said construct is administered as a complex of said construct and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

179. The method of claim 178, wherein said one or more cationic compounds are one or more cationic lipids.

180. The method of claim 179, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide , dipalmitoyl-phophatidylethanolamine 5-carboxy-spermylamide, {3β-[N-N',N'-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-

dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

181. The method of claim 179, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

182. The method of claim 179, wherein said complex further comprising one or more neutral lipids.

183. The method of claim 179, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

184. The method of claim 182, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

185. The method of claim 184, wherein the ratio of said polynucleotide construct to lipid is about 5:1 to about 1:1.

186. The method of claim 155, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4151 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

187. The method of claim 155, wherein said polynucleotide construct is VR4112 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-

dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4112 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

188. The method of claim 155, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR1103 (SEQ ID No. 25) to lipid is from about 5:1 to about 1:1.

189. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding interferon ω is administered to treat ovarian carcinoma.

190. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding interferon ω is administered to treat metastasis of ovarian carcinoma.

191. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding interleukin-2 is administered to treat ovarian carcinoma.

192. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding interleukin-2 is administered to treat metastasis of ovarian carcinoma.

193. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding an interleukin-2 is administered to treat melanoma.

194. The method of claim 156, wherein a polynucleotide construct comprising a DNA molecule encoding an interleukin-2 is administered to treat metastasis of melanoma.

195. The method of claim 155, wherein said polynucleotide is DNA operably linked to a promoter.

196. The method of claim 155, wherein said polynucleotide is RNA.

197. The method of claim 155, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional molecule.

198. The method of claim 155, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional cytokines.

199. The method of claim 155, wherein said polynucleotide comprises a region regulating gene expression.

200. The method of claim 199, wherein said region regulating gene expression is cell specific or tissue specific.

201. The method of claim 200, wherein said region is tumor cell or tumor tissue specific.

202. A method of treating cancer, or metastasis thereof, in a mammal, comprising:

the method of claim 155 in combination with one or more additional cancer treatment methods selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, and gene therapy.

203. The method of claim 202, wherein said polynucleotide construct is administered prior to the commencement of said one or more additional cancer treatment methods.

204. The method of claim 202, wherein said polynucleotide construct is administered during the practice of said at one or more additional cancer treatment methods.

205. The method of claim 202, wherein said polynucleotide construct is administered after the end of said one or more additional cancer treatment methods.

206. A method of selectively transfecting malignant cells in a body cavity of a mammal, comprising:

administering into a body cavity of said mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a molecule, or an active fragment thereof, such that said molecule is delivered substantially to and expressed in malignant cells within said body cavity.

207. The method of claim 206, wherein said molecule is a peptide, polypeptide or protein.

208. The method of claim 206, wherein said molecule is cytoplasmic, nuclear, secreted, or membrane bound.

209. The method of claim 206, wherein said molecule induceswherein said molecule induces rejection, regression or prevents progression of said malignant cells in the mammal.

210. The method of claim 206, wherein said molecule is immunogenic.

211. The method of claim 206, wherein said polynucleotide encodes at least one member selected from the group consisting of cytokine, lymphokine Class 1 or II histocompatibililty antigens, costimulatory molecules, bacterial antigen, viral glycoprotein, lysozyme, recombinant antibody, adhesion molecule, toxic peptide, and tumor suppressor.

212. The method of 206, wherein said molecule is a cytokine.

213. The method of 212, wherein said cytokine is selected from the group consisting IFNω, IFNα, IFNτ, IFNγ, IFNβ, IL-1, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GM-CSF, an active fragment of any of thereof, and a combination of any of thereof.

214. The method of claim 213, wherein said cytokine is an interferon ω.

215. The method of claim 214, wherein said interferon ω is a polypeptide comprising amino acids -23 to 172 in SEQ ID No. 8.

216. The method of claim 214, wherein said interferon ω is a polypeptide comprising amino acids 1 to 172 in SEQ ID No. 8.

217. The method of claim 214, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4).

218. The method of claim 213, wherein said cytokine is an interferon α.

219. The method of claim 218, wherein interferon α is a polypeptide comprising amino acids -23 to 166 of SEQ ID No. 10.

220. The method of claim 218, wherein interferon α is a polypeptide comprising amino acids 1 to 166 of SEQ ID No. 10.

221. The method of claim 218, wherein said polynucleotide construct is VR4112 (SEQ ID No. 2).

222. The method of claim 213, wherein said cytokine is an interleukin-2.

223. The method of claim 222, wherein interleukin-2 is a polypeptide comprising amino acids 58 to 105 of SEQ ID No. 14.

224. The method of claim 222, wherein interleukin-2 is a polypeptide comprising amino acids 20 to 126 of SEQ ID No. 14.

225. The method of claim 222, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25).

226. The method of claim 206, wherein said malignant cells form a cancer in the mammal.

227. The method of claim 226, wherein said molecule is encoded in an amount sufficient to suppress the dissemination of said malignant cells within said body cavity.

228. The method of claim 206, wherein the body cavity is selected from the group consisting of peritoneal cavity, thoracic cavity, plural cavity, rectal cavity, stomach cavity, and urinary bladder vesicle.

229. The method of claim 228, wherein the body cavity is peritoneal cavity.

230. The method of claim 227, wherein said cancer is selected from the group consisting of renal cell carcinoma, colorectal carcinoma, lymphoma, melanoma, prostate cancer, ovarian cancer, lung cancer, stomach cancer, glandular lymphoma, bladder cancer, uterine cancer, mesenteric cancer, pancreatic cancer, gastric cancer, metastases thereof, and combinations thereof.

231. The method of claim 226, wherein said cancer is pancreatic, gasric, colon, ovarian, or mesenteric cancer.

232. The method of claim 206, wherein said administering is by injection or infusion.

233. The method of claim 206, wherein said polynucleotide construct is administered free from *ex vivo* cells or *ex vivo* cellular material.

234. The method of claim 206, wherein said construct is targeted to a tumor.

235. The method of claim 206, wherein said construct is injected intraperitoneally.

236. The method of claim 206, wherein said mammal is a human.

237. The method of claim 206, wherein said construct is free from association with transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

238. The method of claim 206, wherein said construct is administered as a complex of said construct and one or more cationic

compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

239. The method of claim 238, wherein said one or more cationic compounds are one or more cationic lipids.

240. The method of claim 239, wherein said cationic lipids are selected from the group consisting of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-l-propaniminium bromide, 5-carboxyspermylglycine dioctadecylamide , dipalmitoyl-phophatidylethanolamine 5-carboxy-spermylamide, {3β-[N-N′,N′-dimethylamino)ethane]-carbomoyl}-cholesterol, dimethyldioctdecyl-ammonium bromide,(±)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, 1-*O*-oleyl-2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium, cationic lipids are 3,5-(NN-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and 3,5-(N,N-di-lysyl)diamino-benzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine) and mixtures thereof.

241. The method of claim 239, wherein said cationic lipids comprise (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide.

242. The method of claim 239, wherein said complex further comprising one or more neutral lipids.

243. The method of claim 239, wherein said neutral lipids comprise 1,2-dioleoyl-glycero-3-phosphoethanolamine.

244. The method of claim 242, wherein said polynucleotide construct is complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine.

245. The method of claim 244, wherein the ratio of said polynucleotide construct to lipid is about 5:1 to about 1:1.

246. The method of claim 206, wherein said polynucleotide construct is VR4151 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4151 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

247. The method of claim 206, wherein said polynucleotide construct is VR4112 (SEQ ID No. 4) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR4112 (SEQ ID No. 4) to lipid is from about 5:1 to about 1:1.

248. The method of claim 206, wherein said polynucleotide construct is VR1103 (SEQ ID No. 25) complexed with (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide and 1,2-dioleoyl-glycero-3-phosphoethanolamine such that the mass ratio of VR1103 (SEQ ID No. 25) to lipid is from about 5:1 to about 1:1.

249. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding interferon ω is administered to treat ovarian carcinoma.

250. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding interferon ω is administered to treat metastasis of ovarian carcinoma.

251. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding interleukin-2 is administered to treat ovarian carcinoma.

252. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding interleukin-2 is administered to treat metastasis of ovarian carcinoma.

253. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding an interleukin-2 is administered to treat melanoma.

254. The method of claim 213, wherein a polynucleotide construct comprising a DNA molecule encoding an interleukin-2 is administered to treat metastasis of melanoma.

255. The method of any one of claim 206, wherein said polynucleotide is DNA operably linked to a promoter.

256. The method of claim 206, wherein said polynucleotide is RNA.

257. The method of claim 206, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional molecule.

258. The method of claim 206, wherein said polynucleotide construct further comprises a polynucleotide encoding one or more additional cytokines.

259. The method of claim 206, wherein said polynucleotide comprises a region regulating gene expression.

260. The method of claim 259, wherein said region regulating gene expression is cell specific or tissue specific.

261. The method of claim 260, wherein said region is tumor cell or tumor tissue specific.

262. A method of treating cancer, or metastasis thereof, in a mammal, comprising:

the method of claim 227 in combination with one or more additional cancer treatment methods selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, and gene therapy.

263. The method of claim 262, wherein said polynucleotide construct is administered prior to the commencement of said one or more additional cancer treatment methods.

264. The method of claim 262, wherein said polynucleotide construct is administered during the practice of said at one or more additional cancer treatment methods.

265. The method of claim 262, wherein said polynucleotide construct is administered after the end of said one or more additional cancer treatment methods.

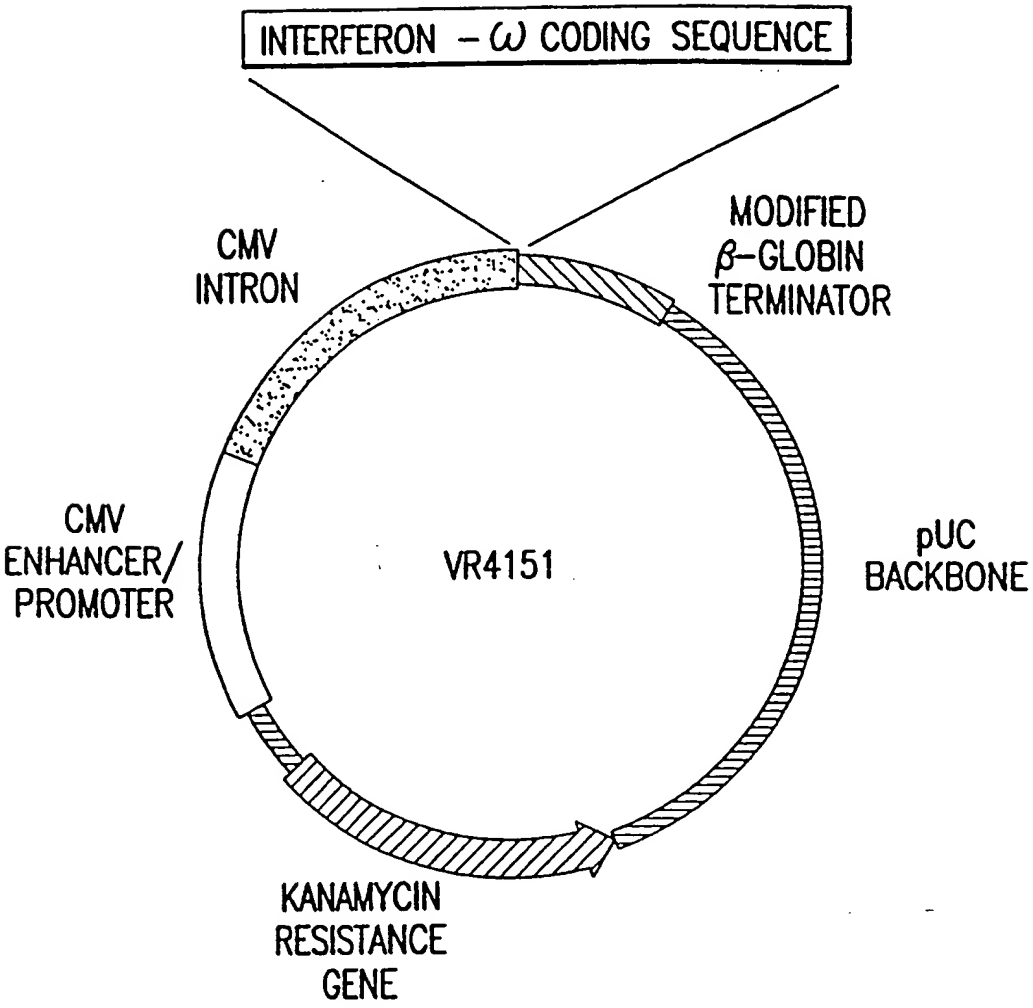
INTERFERON – ω CODING SEQUENCE
CMV INTRON
MODIFIED β–GLOBIN TERMINATOR
CMV ENHANCER/ PROMOTER
VR4151
pUC BACKBONE
KANAMYCIN RESISTANCE GENE

FIG. 1

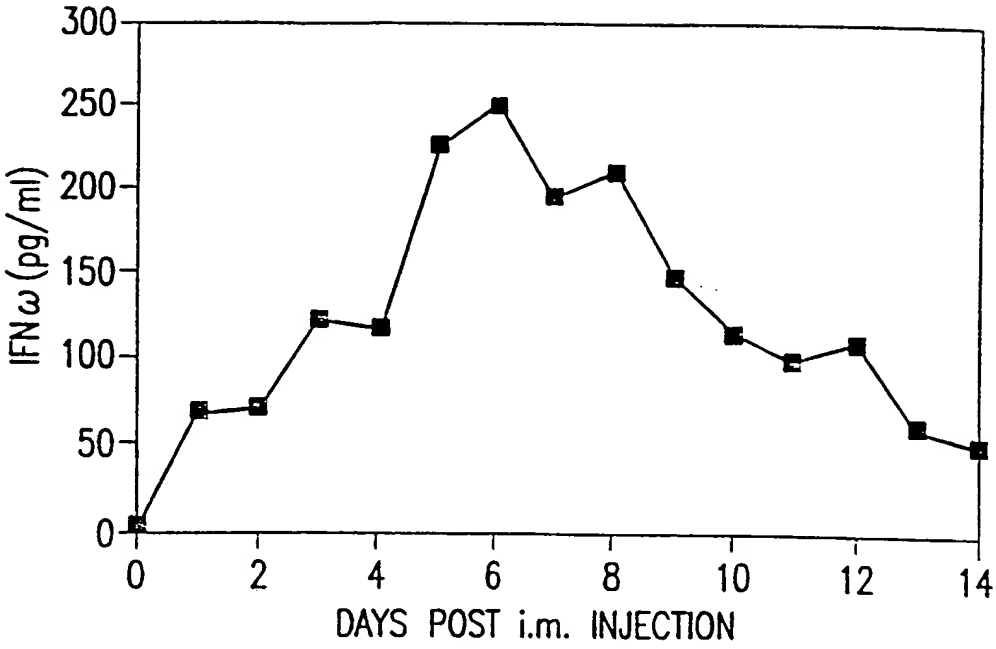
300
250
200
150
100
50
0
IFN ω (pg/ml)
0
2
4
6
8
10
12
14
DAYS POST i.m. INJECTION

FIG.2A

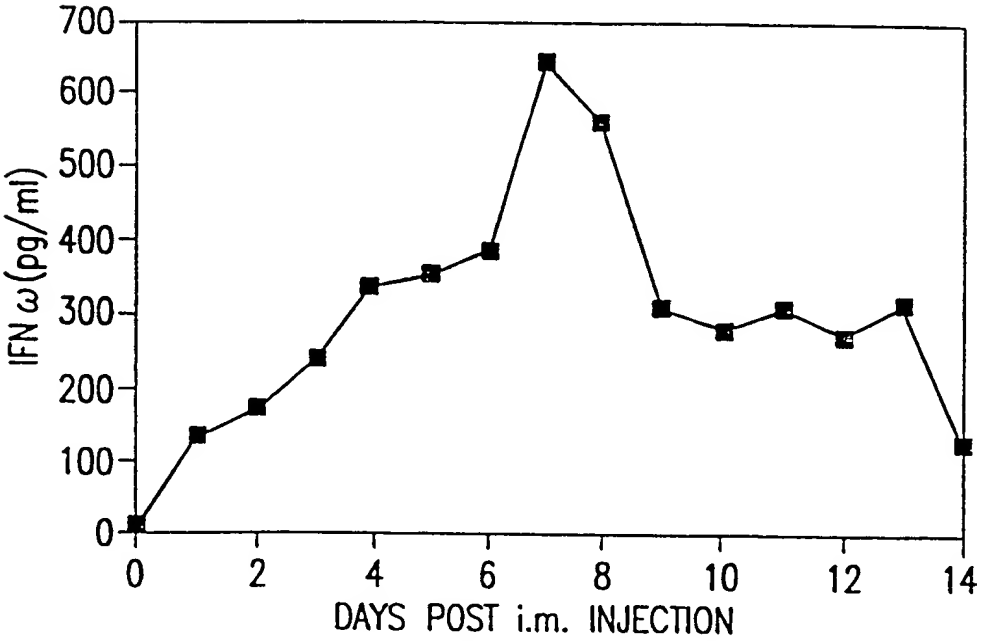
700
600
500
400
300
200
100
0
IFN ω (pg/ml)
0
2
4
6
8
10
12
14
DAYS POST i.m. INJECTION

FIG.2B

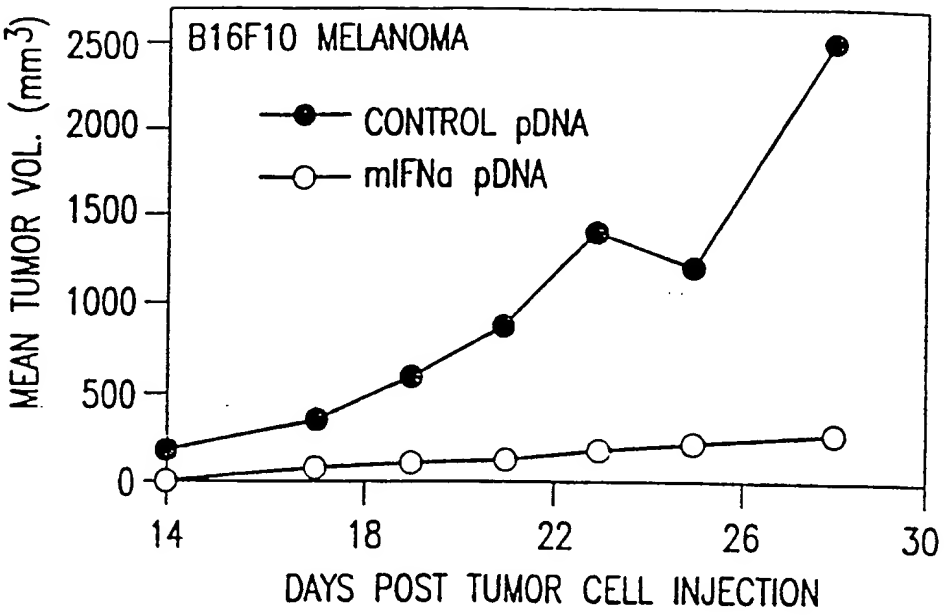
B16F10 MELANOMA
CONTROL pDNA
mIFNα pDNA
MEAN TUMOR VOL. (mm3)
2500
2000
1500
1000
500
0
14
18
22
26
30
DAYS POST TUMOR CELL INJECTION

FIG.3A

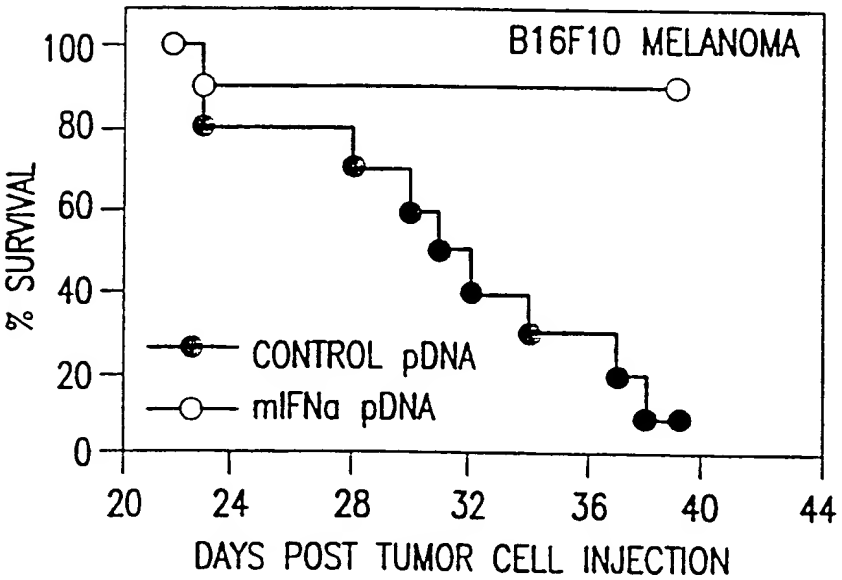
B16F10 MELANOMA
CONTROL pDNA
mIFNα pDNA
% SURVIVAL
100
80
60
40
20
0
20
24
28
32
36
40
44
DAYS POST TUMOR CELL INJECTION

FIG.3B

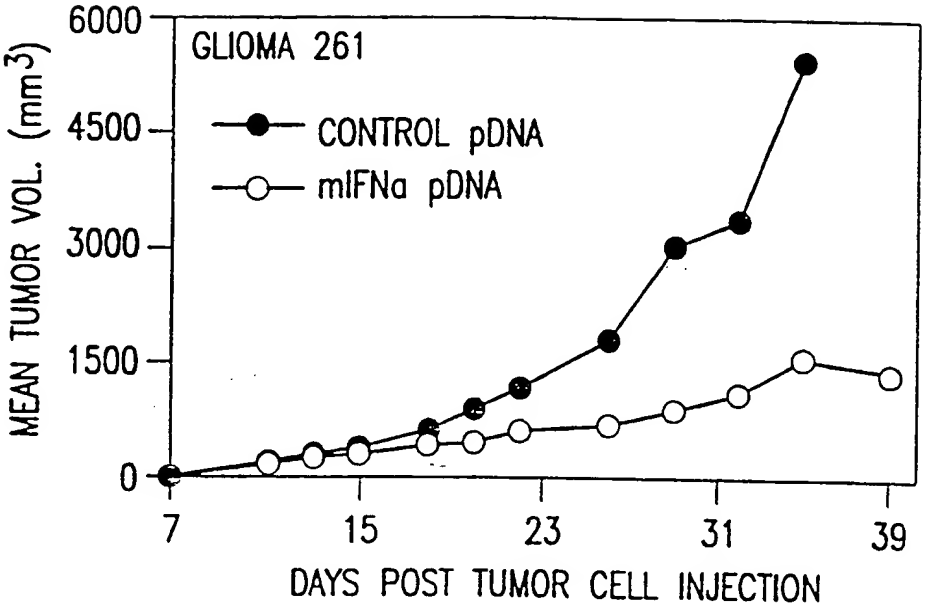
GLIOMA 261
CONTROL pDNA
mIFNα pDNA
MEAN TUMOR VOL. (mm3)
6000
4500
3000
1500
0
7
15
23
31
39
DAYS POST TUMOR CELL INJECTION

FIG.3C

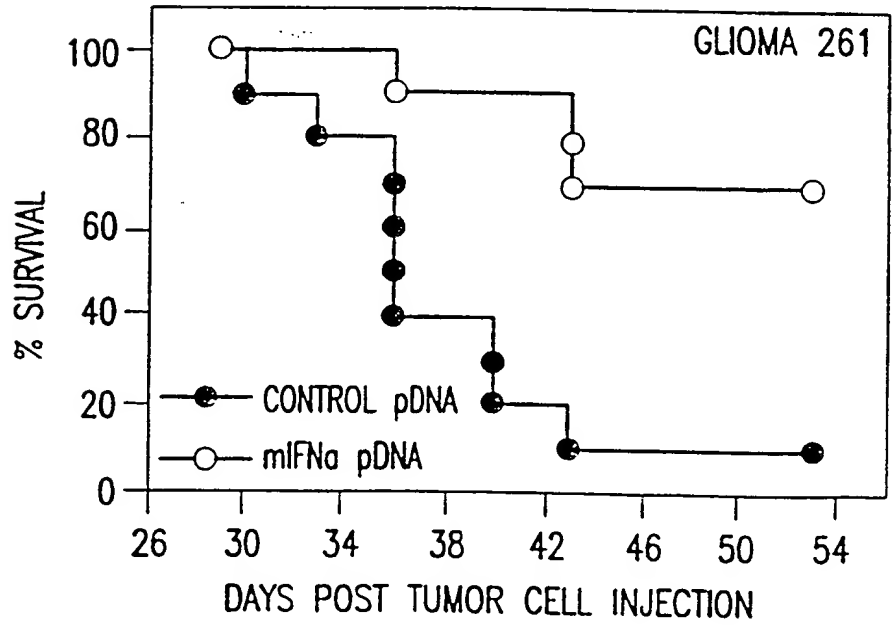
GLIOMA 261
CONTROL pDNA
mIFNα pDNA
% SURVIVAL
100
80
60
40
20
0
26
30
34
38
42
46
50
54
DAYS POST TUMOR CELL INJECTION

FIG.3D

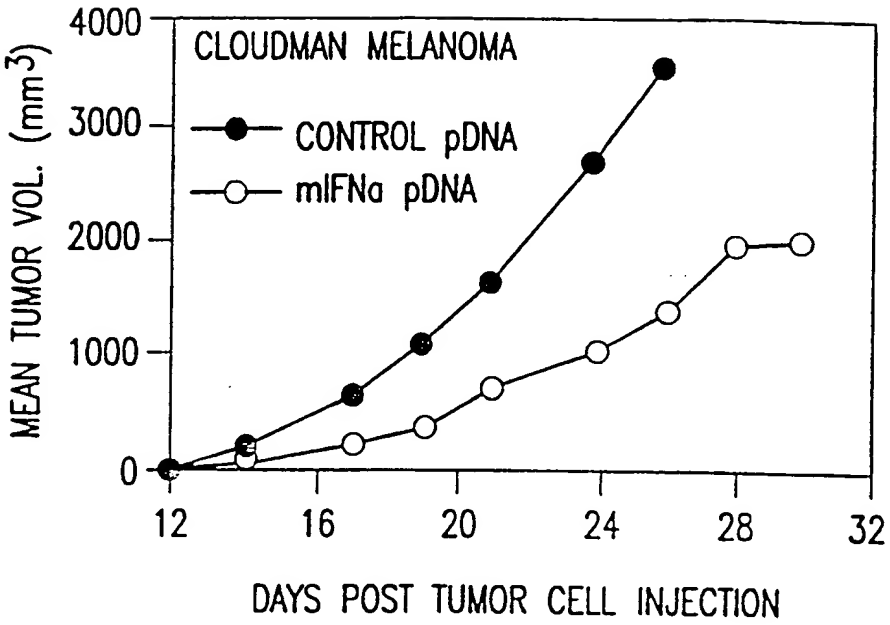
CLOUDMAN MELANOMA
CONTROL pDNA
mIFNα pDNA
MEAN TUMOR VOL. (mm3)
4000
3000
2000
1000
0
12
16
20
24
28
32
DAYS POST TUMOR CELL INJECTION

FIG.3E

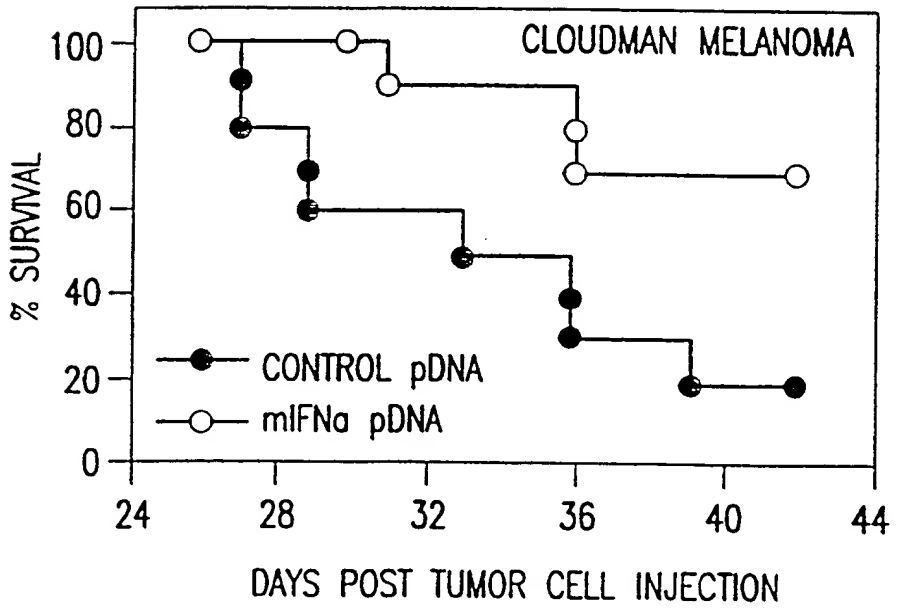
CLOUDMAN MELANOMA
CONTROL pDNA
mIFNα pDNA
% SURVIVAL
100
80
60
40
20
0
24
28
32
36
40
44
DAYS POST TUMOR CELL INJECTION

FIG.3F

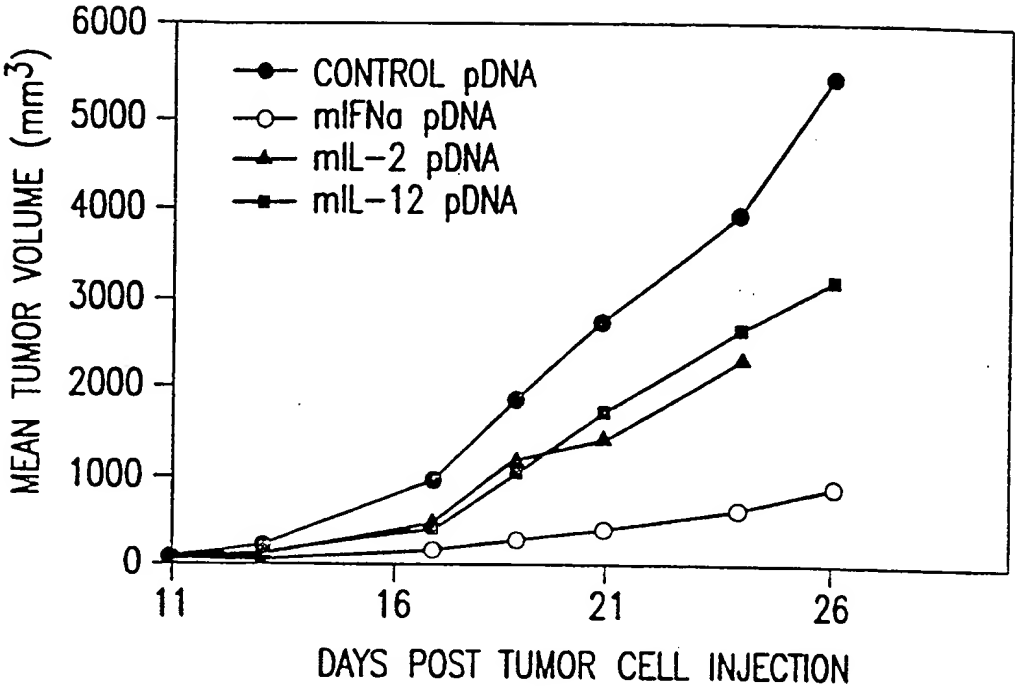
6000
5000
4000
3000
2000
1000
0
MEAN TUMOR VOLUME (mm3)
CONTROL pDNA
mIFNα pDNA
mIL-2 pDNA
mIL-12 pDNA
11
16
21
26
DAYS POST TUMOR CELL INJECTION

FIG.4A

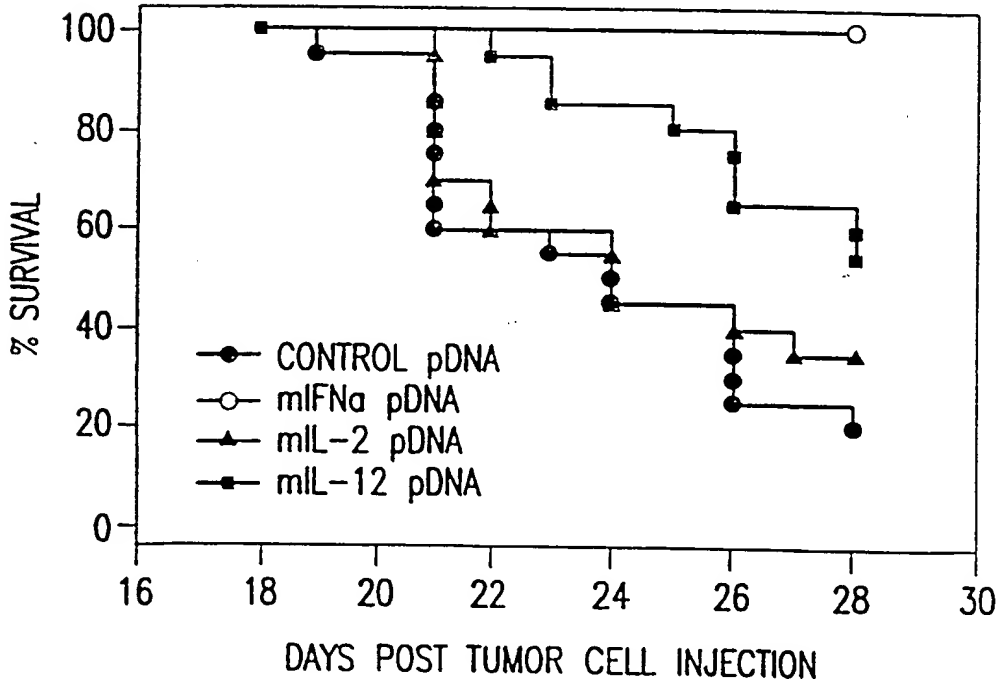
100
80
60
40
20
0
% SURVIVAL
CONTROL pDNA
mIFNα pDNA
mIL-2 pDNA
mIL-12 pDNA
16
18
20
22
24
26
28
30
DAYS POST TUMOR CELL INJECTION

FIG.4B

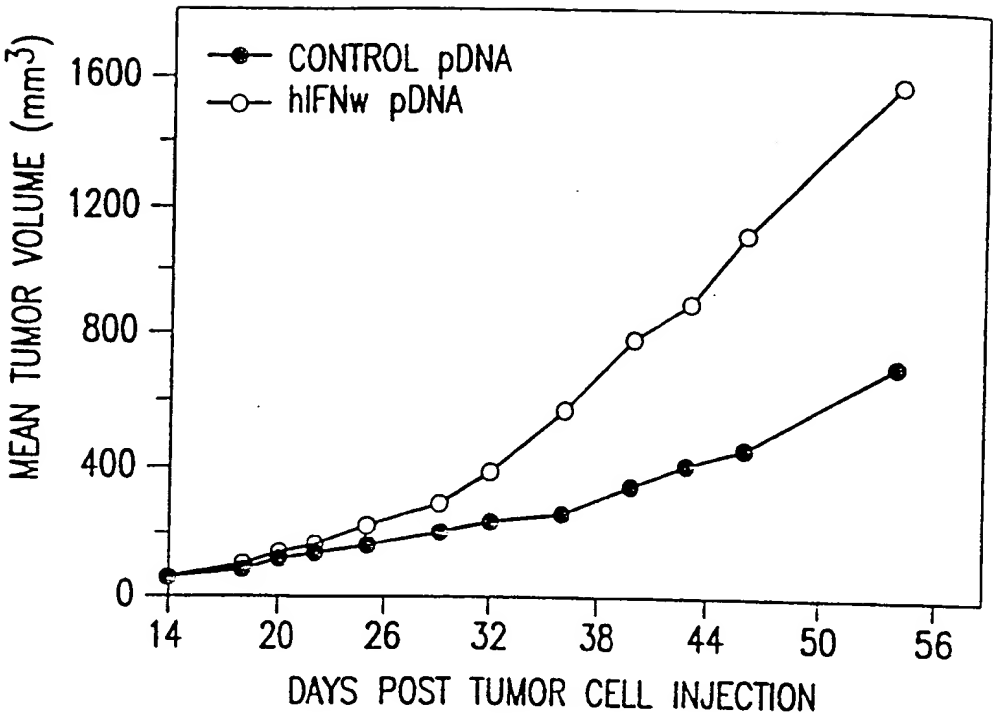
MEAN TUMOR VOLUME (mm^3)
1600
1200
800
400
0
CONTROL pDNA
hIFNw pDNA
14
20
26
32
38
44
50
56
DAYS POST TUMOR CELL INJECTION

FIG.5A

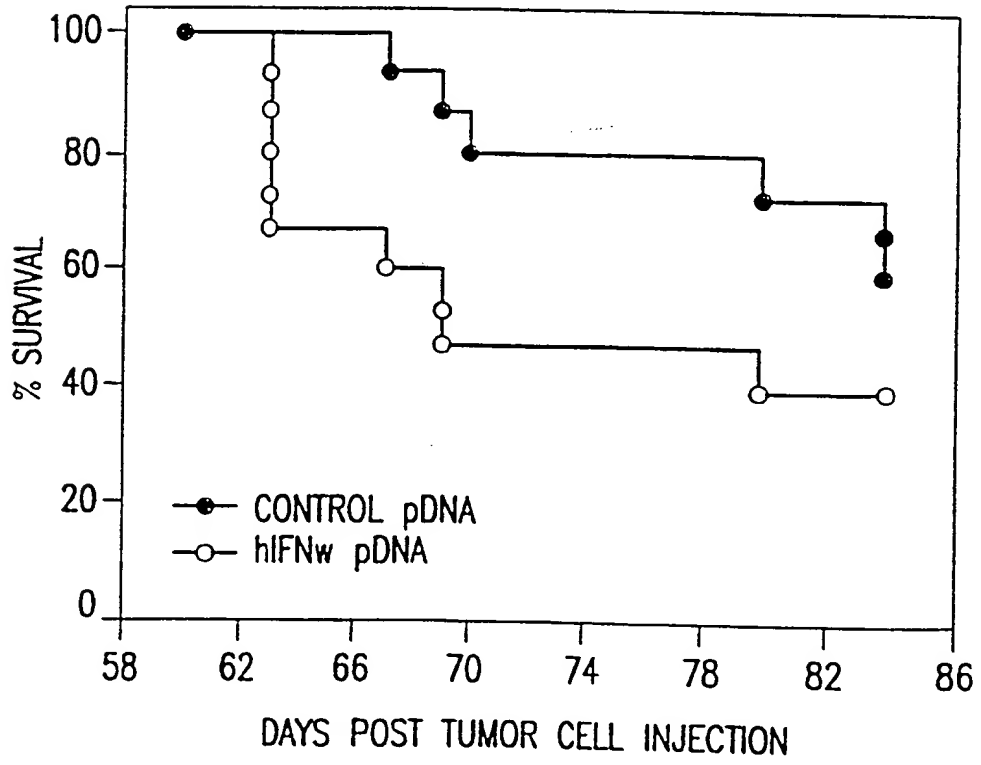
% SURVIVAL
100
80
60
40
20
0
CONTROL pDNA
hIFNw pDNA
58
62
66
70
74
78
82
86
DAYS POST TUMOR CELL INJECTION

FIG.5B

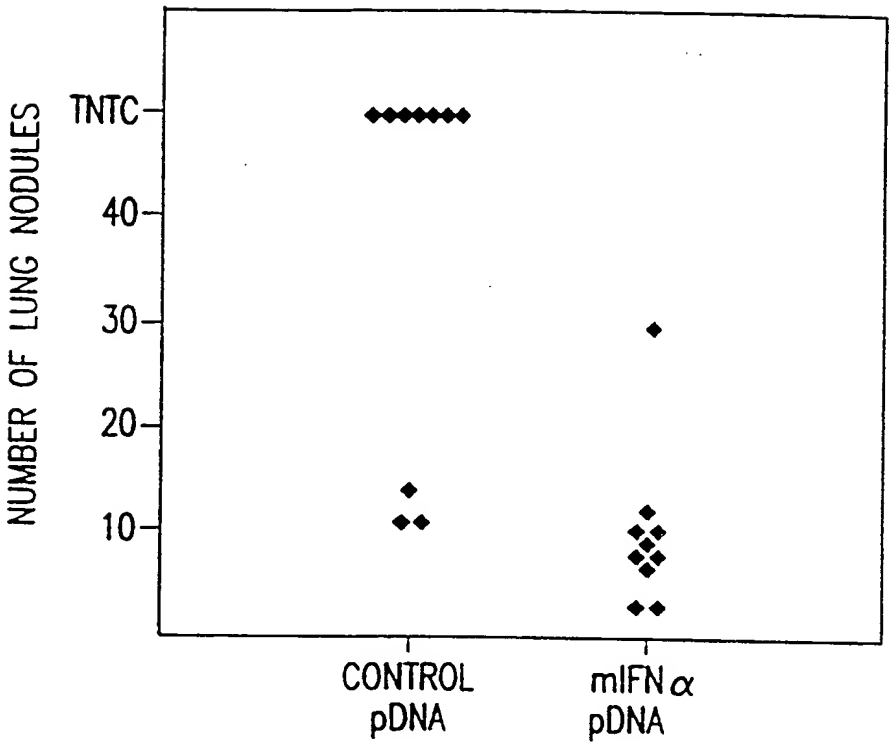
NUMBER OF LUNG NODULES
TNTC
40
30
20
10
CONTROL pDNA
mIFN α pDNA

FIG.6

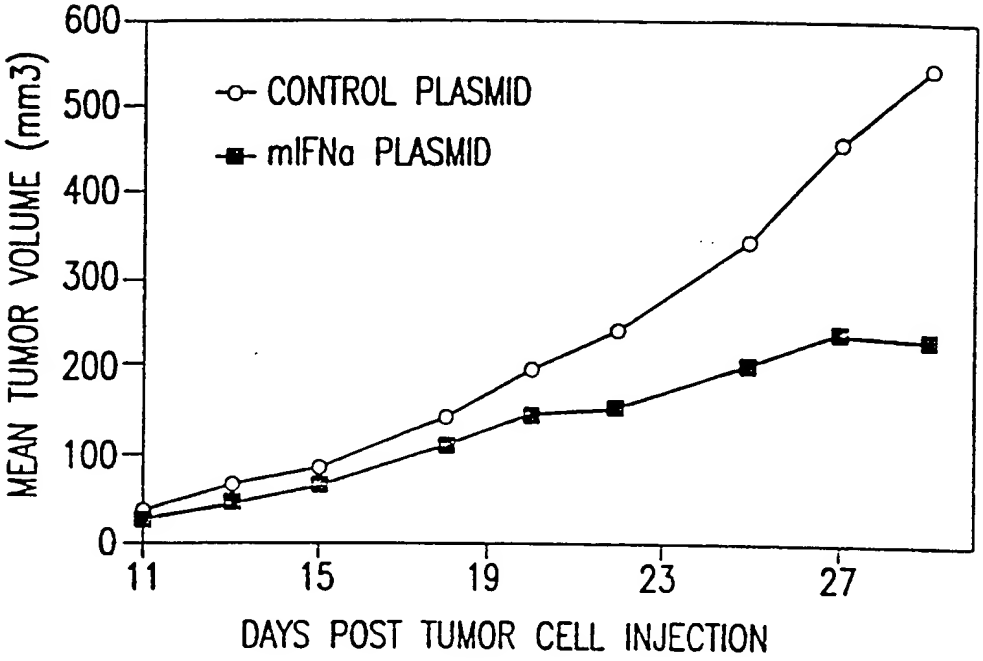
600
500
400
300
200
100
0
MEAN TUMOR VOLUME (mm3)
CONTROL PLASMID
mIFNα PLASMID
11
15
19
23
27
DAYS POST TUMOR CELL INJECTION

FIG.7A

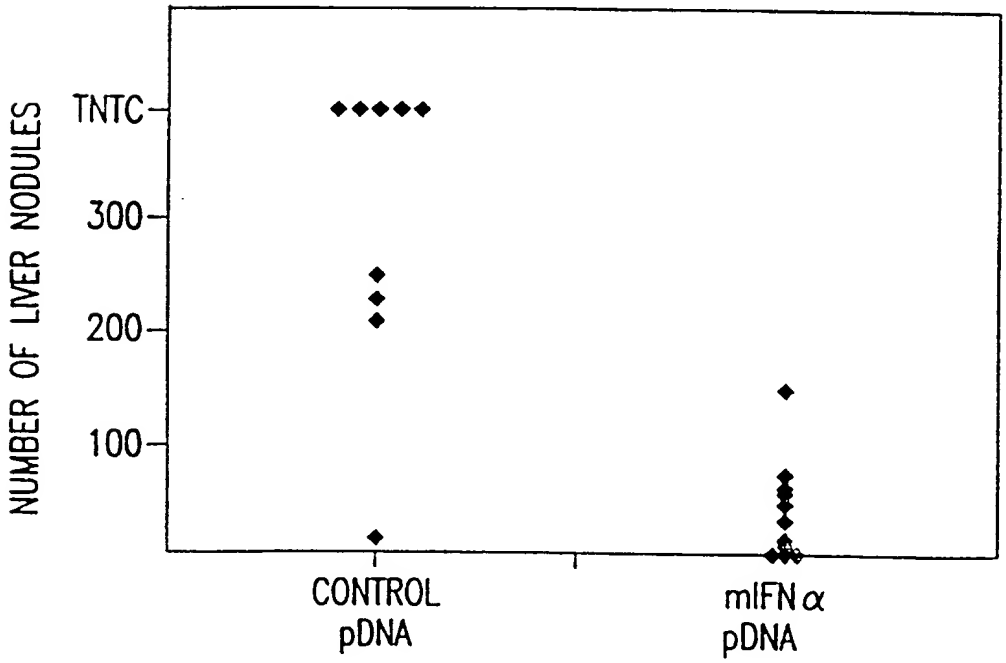
TNTC
300
200
100
NUMBER OF LIVER NODULES
CONTROL pDNA
mIFNα pDNA

FIG.7B

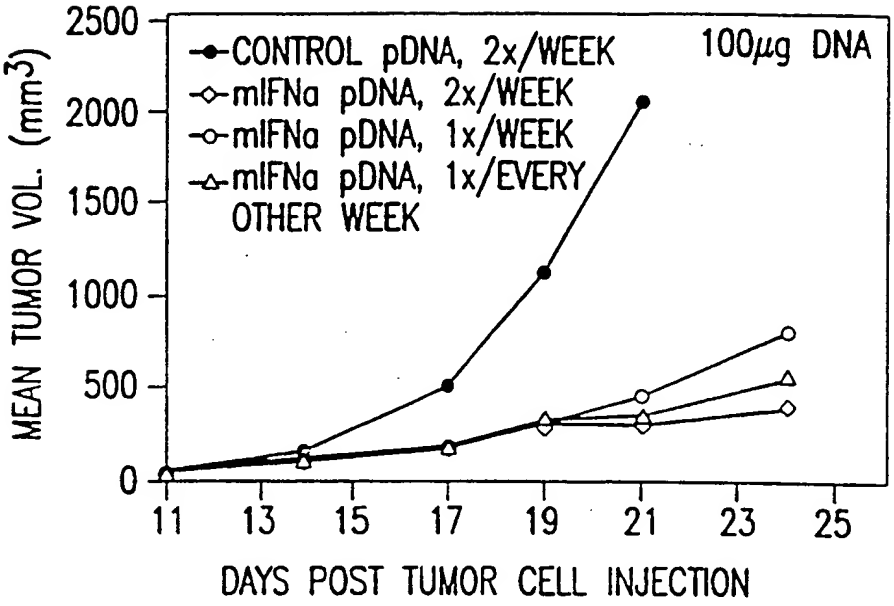
2500
2000
1500
1000
500
0
MEAN TUMOR VOL. (mm3)
CONTROL pDNA, 2x/WEEK
mIFNα pDNA, 2x/WEEK
mIFNα pDNA, 1x/WEEK
mIFNα pDNA, 1x/EVERY OTHER WEEK
100μg DNA
11
13
15
17
19
21
23
25
DAYS POST TUMOR CELL INJECTION

FIG.8A

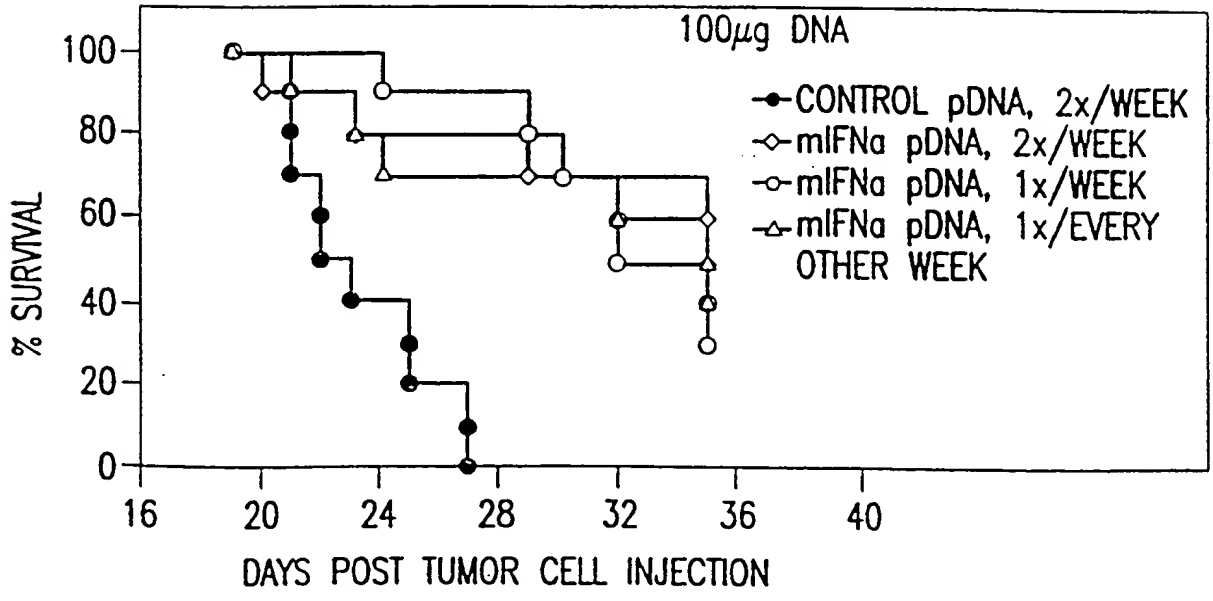
100
80
60
40
20
0
% SURVIVAL
100μg DNA
CONTROL pDNA, 2x/WEEK
mIFNα pDNA, 2x/WEEK
mIFNα pDNA, 1x/WEEK
mIFNα pDNA, 1x/EVERY OTHER WEEK
16
20
24
28
32
36
40
DAYS POST TUMOR CELL INJECTION

FIG.8B

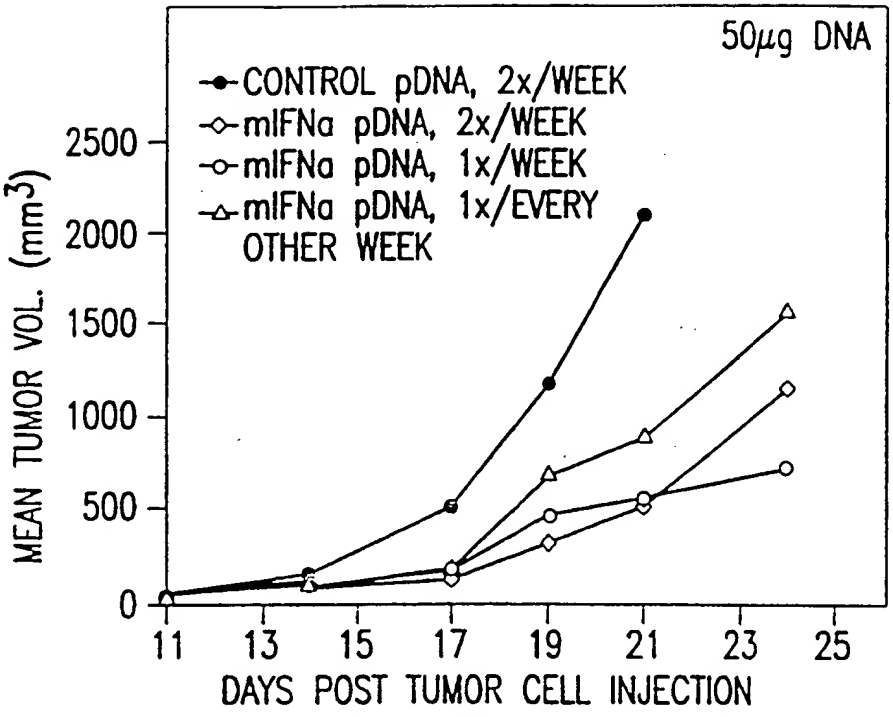
50μg DNA
CONTROL pDNA, 2x/WEEK
mIFNα pDNA, 2x/WEEK
mIFNα pDNA, 1x/WEEK
mIFNα pDNA, 1x/EVERY OTHER WEEK
MEAN TUMOR VOL. (mm3)
2500
2000
1500
1000
500
0
11
13
15
17
19
21
23
25
DAYS POST TUMOR CELL INJECTION

FIG.8C

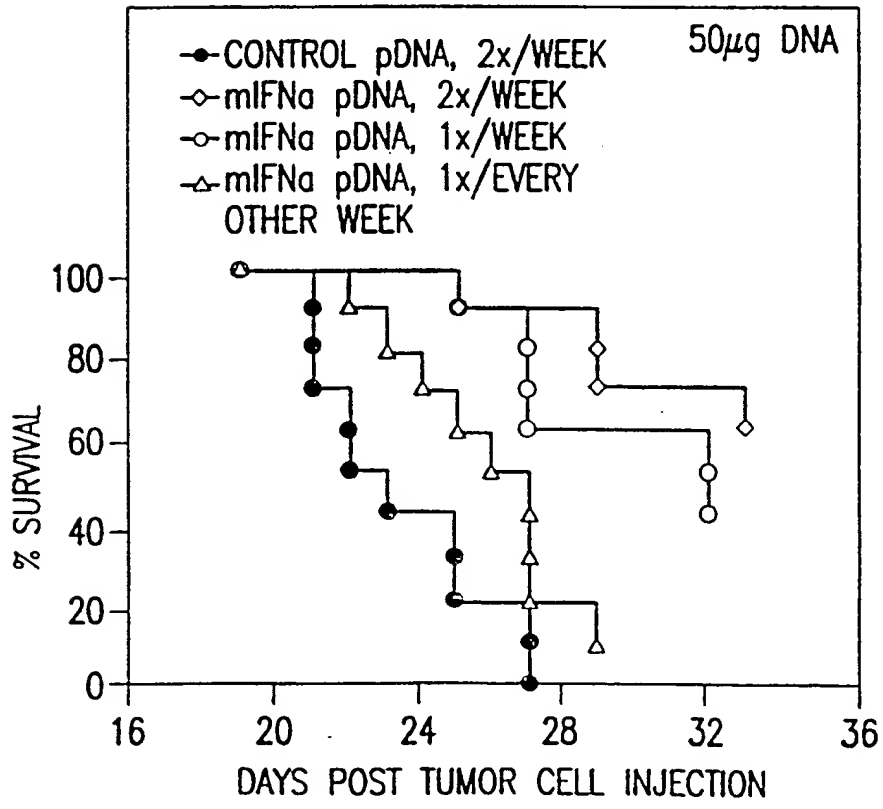
50μg DNA
CONTROL pDNA, 2x/WEEK
mIFNα pDNA, 2x/WEEK
mIFNα pDNA, 1x/WEEK
mIFNα pDNA, 1x/EVERY OTHER WEEK
% SURVIVAL
100
80
60
40
20
0
16
20
24
28
32
36
DAYS POST TUMOR CELL INJECTION

FIG.8D

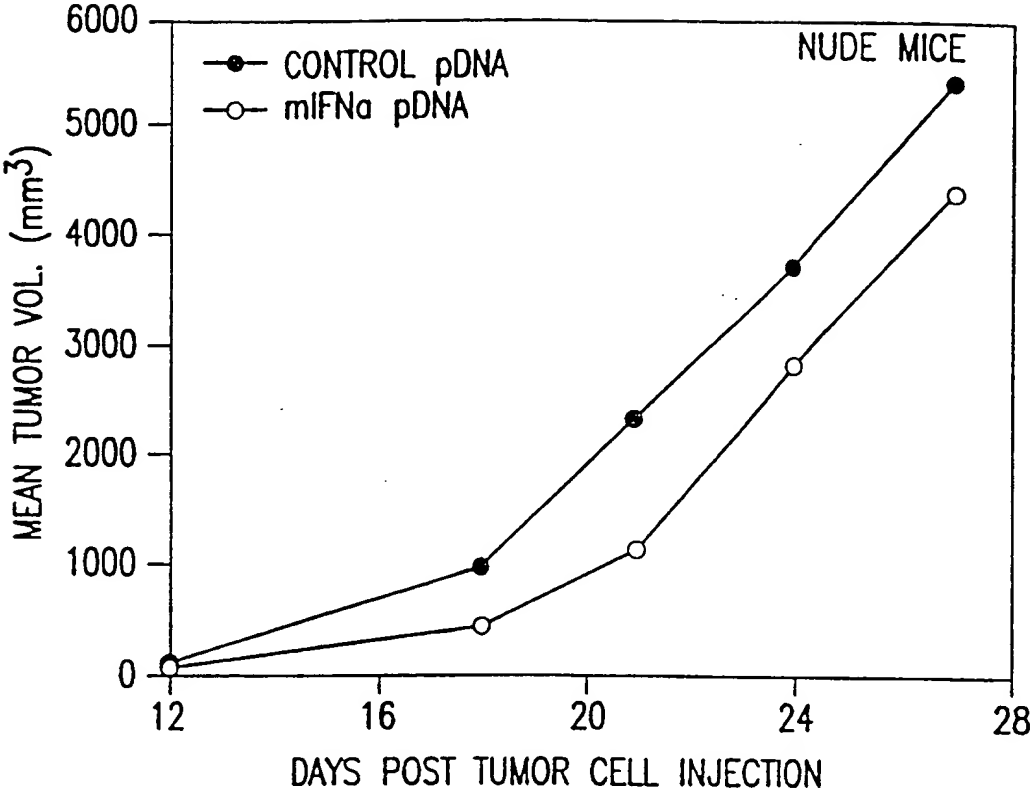
NUDE MICE
CONTROL pDNA
mIFNα pDNA
MEAN TUMOR VOL. (mm3)
6000
5000
4000
3000
2000
1000
0
12
16
20
24
28
DAYS POST TUMOR CELL INJECTION

FIG.9A

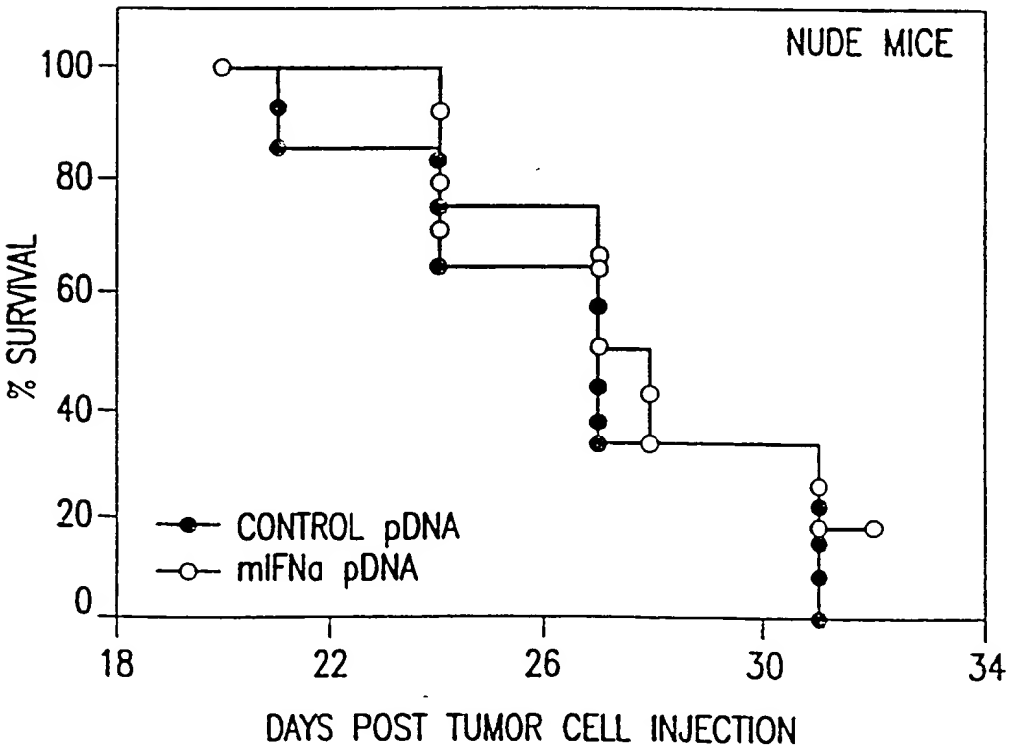
NUDE MICE
% SURVIVAL
100
80
60
40
20
0
CONTROL pDNA
mIFNα pDNA
18
22
26
30
34
DAYS POST TUMOR CELL INJECTION

FIG.9B

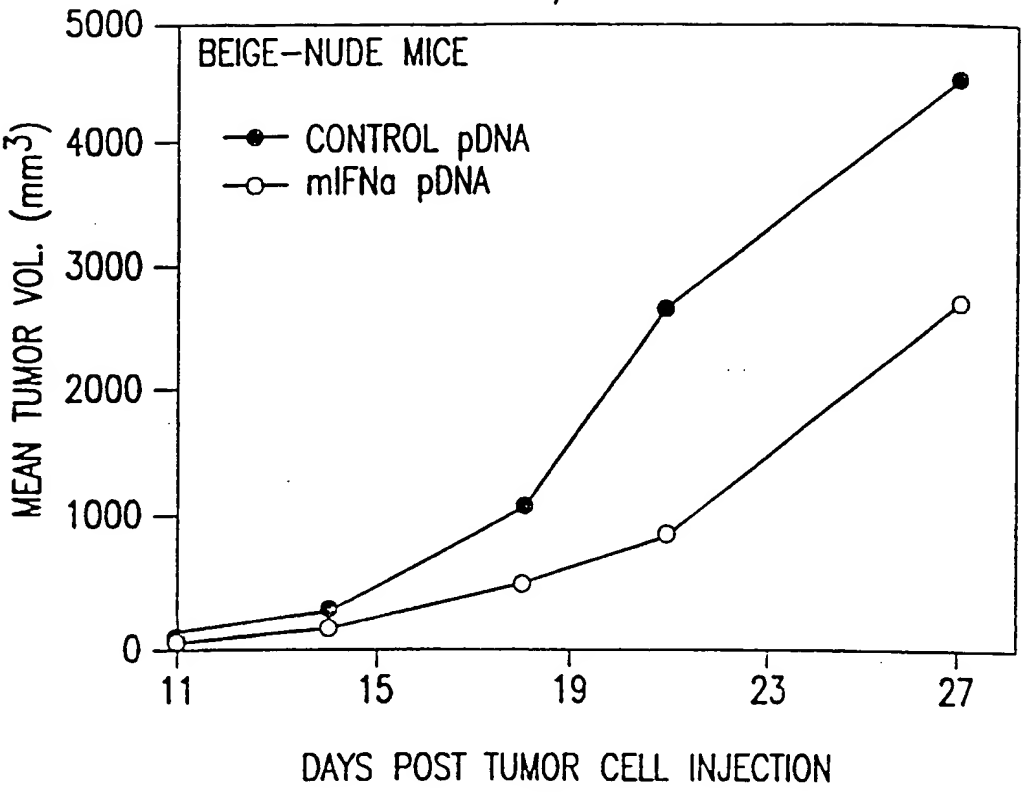
BEIGE-NUDE MICE
CONTROL pDNA
mIFNα pDNA
MEAN TUMOR VOL. (mm3)
5000
4000
3000
2000
1000
0
11
15
19
23
27
DAYS POST TUMOR CELL INJECTION

FIG.9C

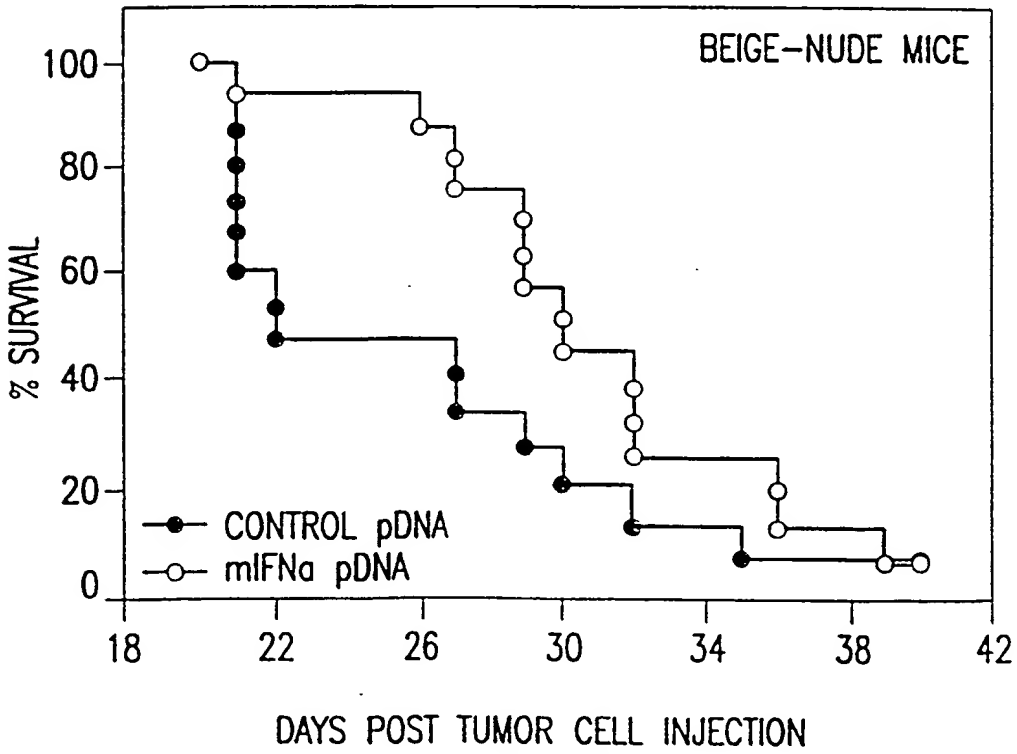
BEIGE-NUDE MICE
CONTROL pDNA
mIFNα pDNA
% SURVIVAL
100
80
60
40
20
0
18
22
26
30
34
38
42
DAYS POST TUMOR CELL INJECTION

FIG.9D

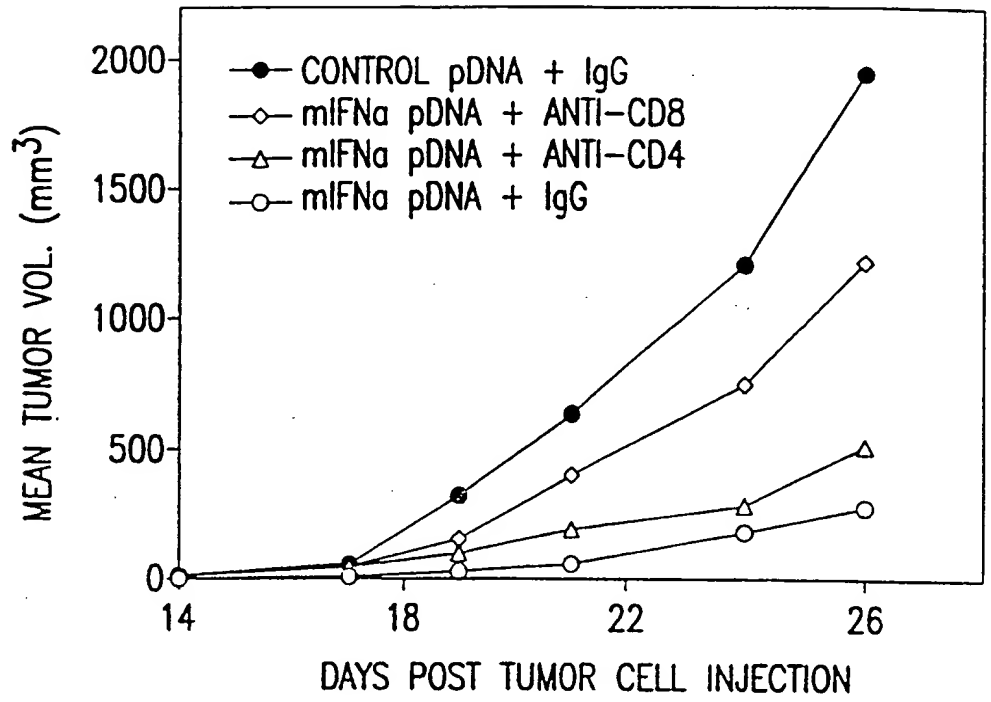

FIG. 10A

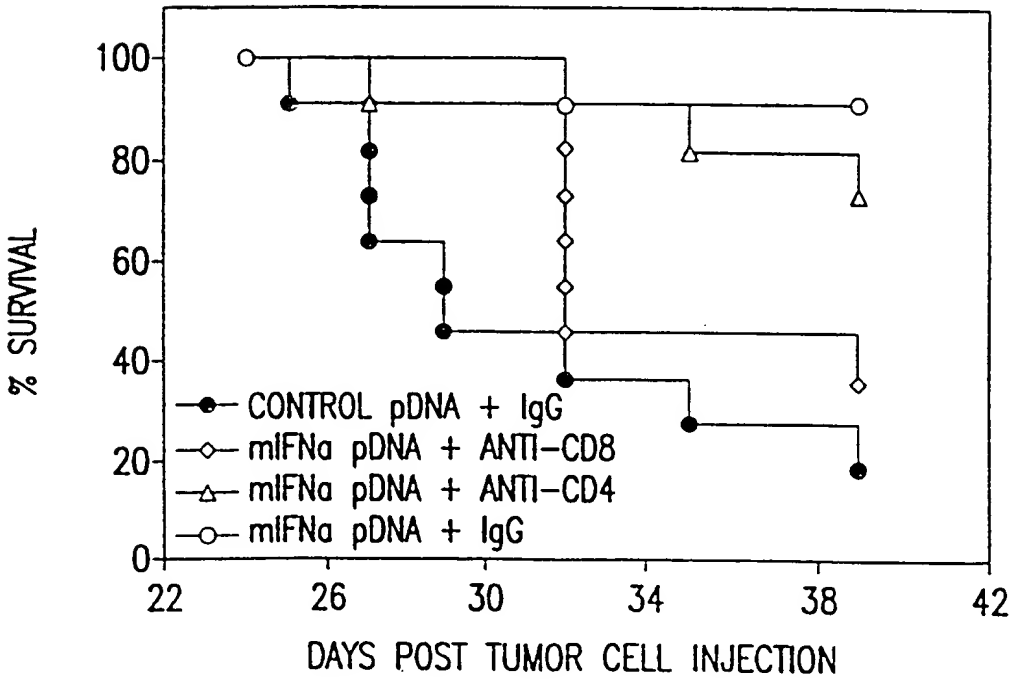

FIG. 10B

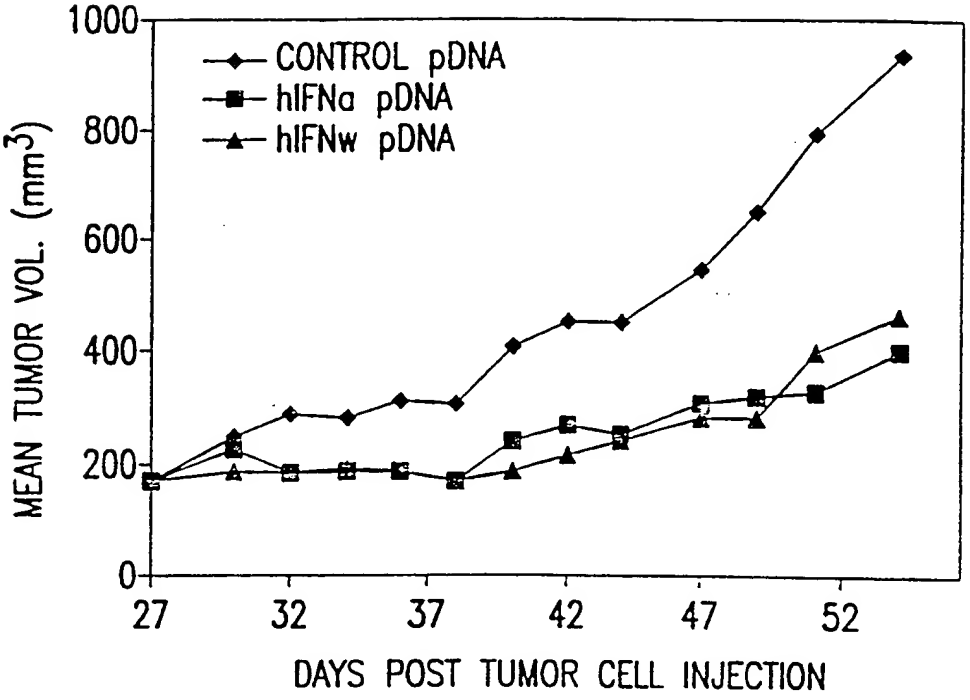

FIG.11A

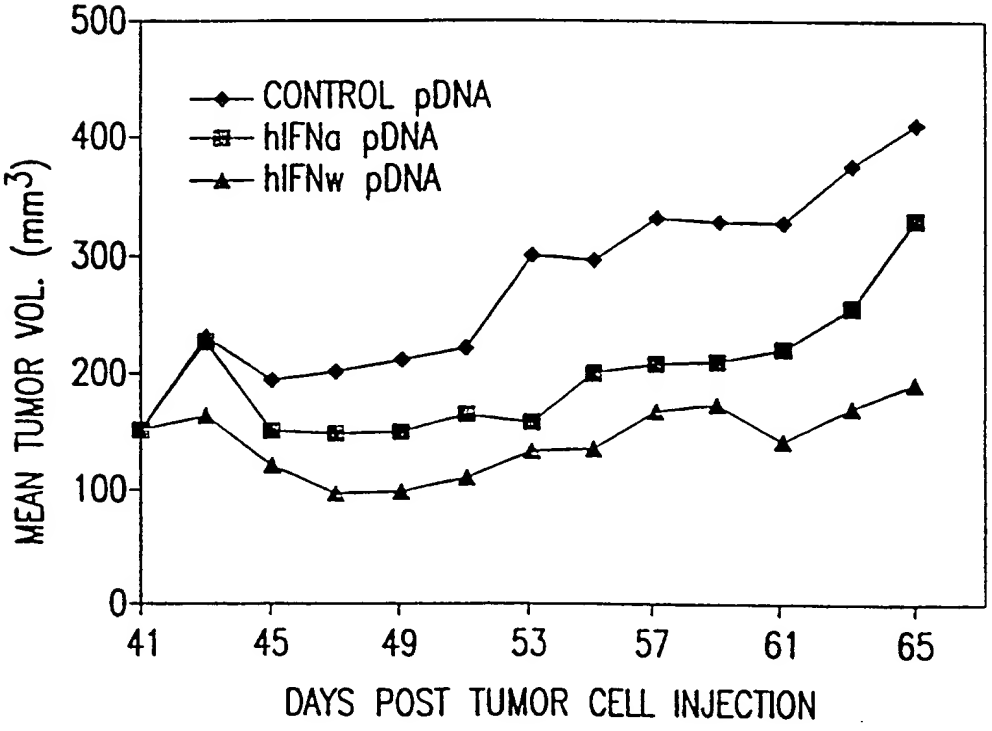

FIG.11B

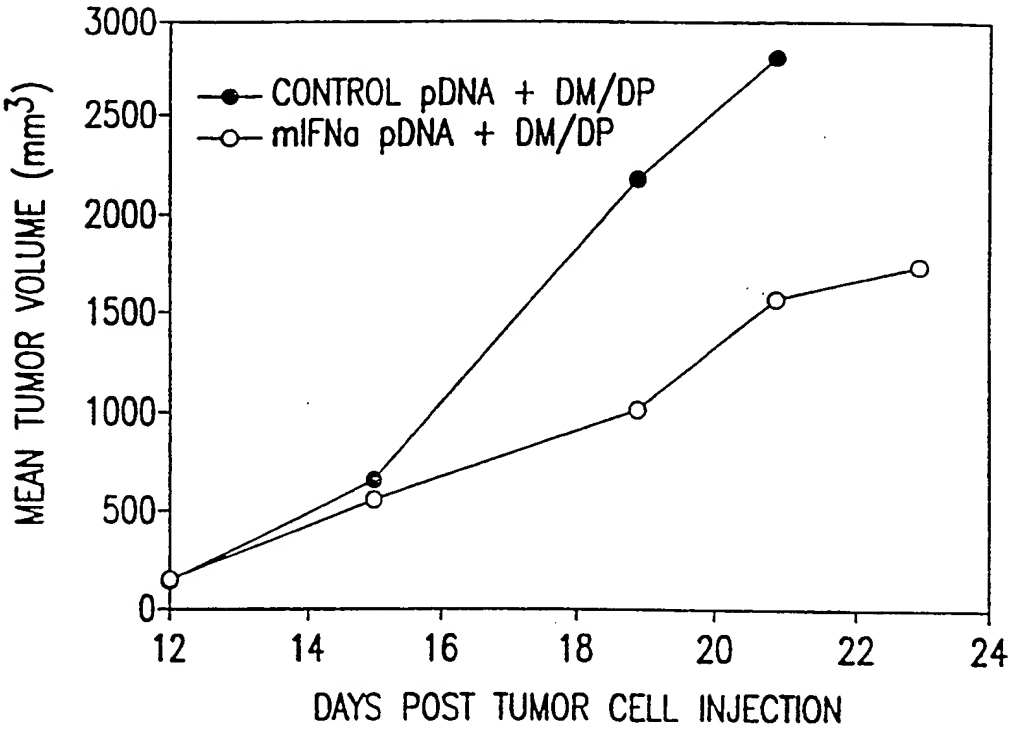

FIG.12A

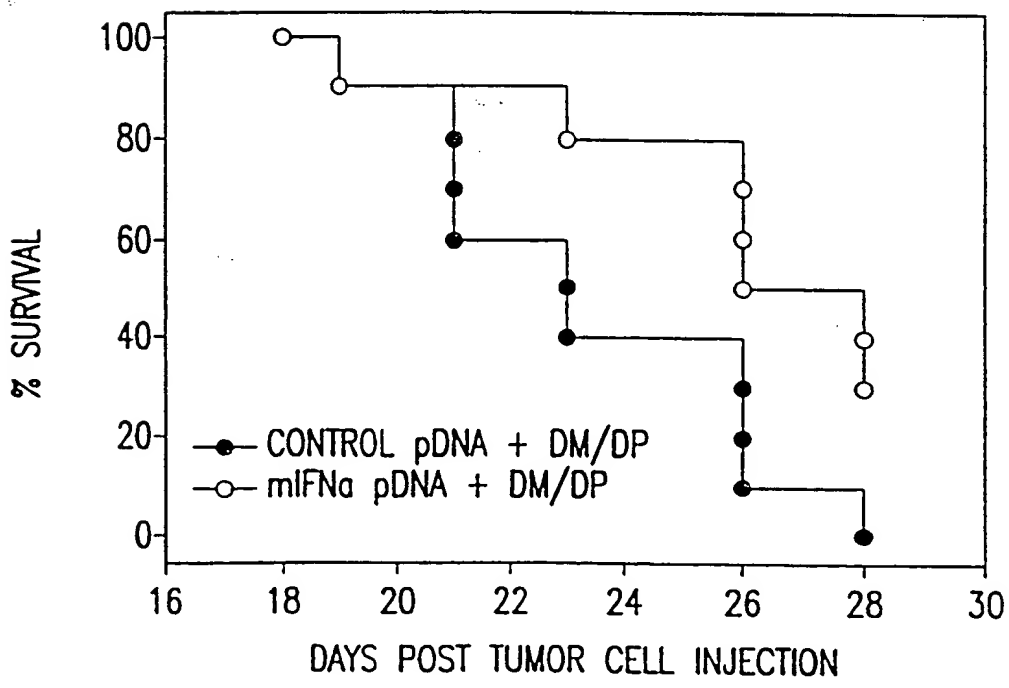

FIG.12B

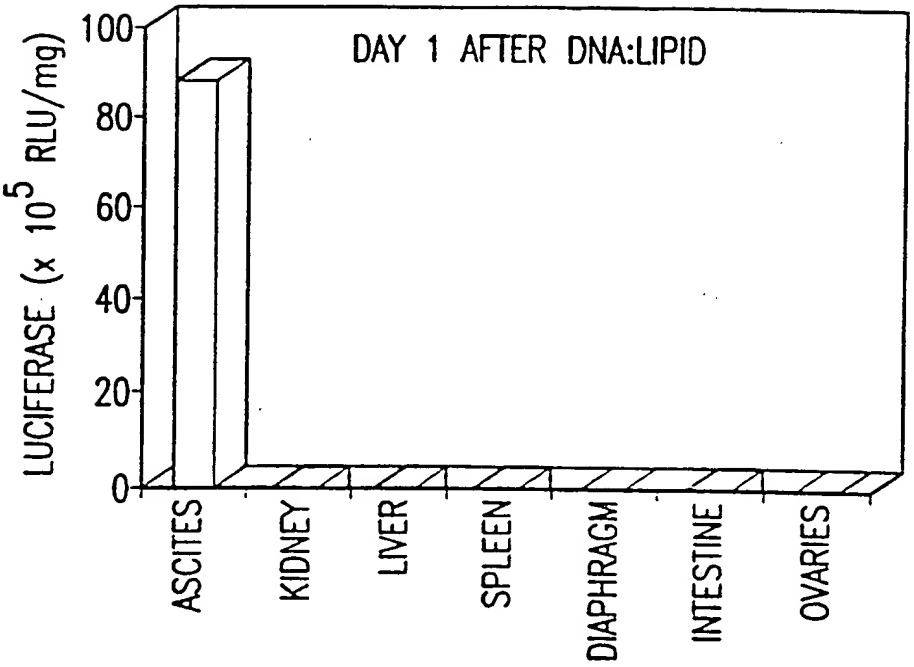

FIG. 13A

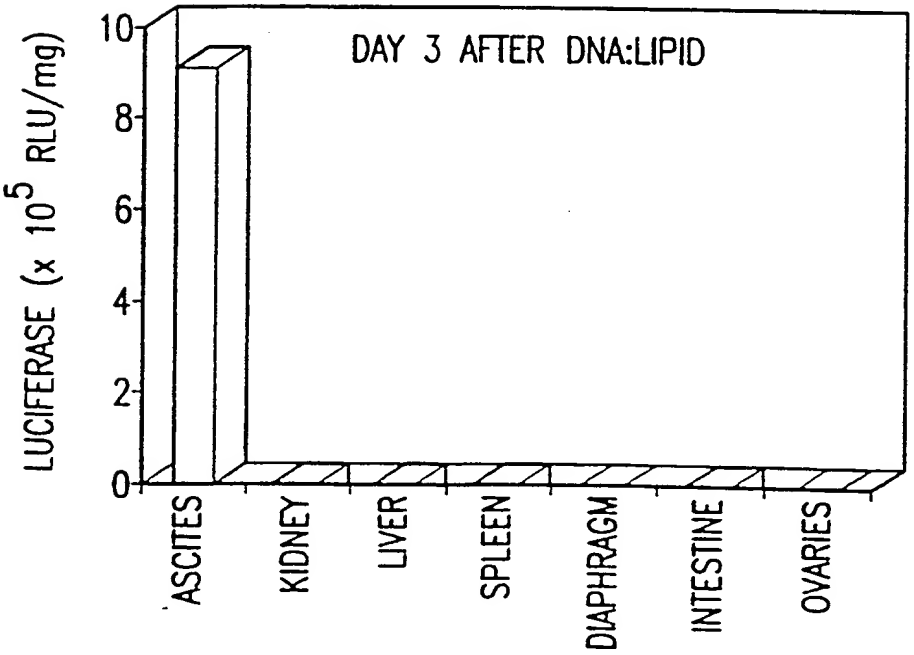

FIG. 13B

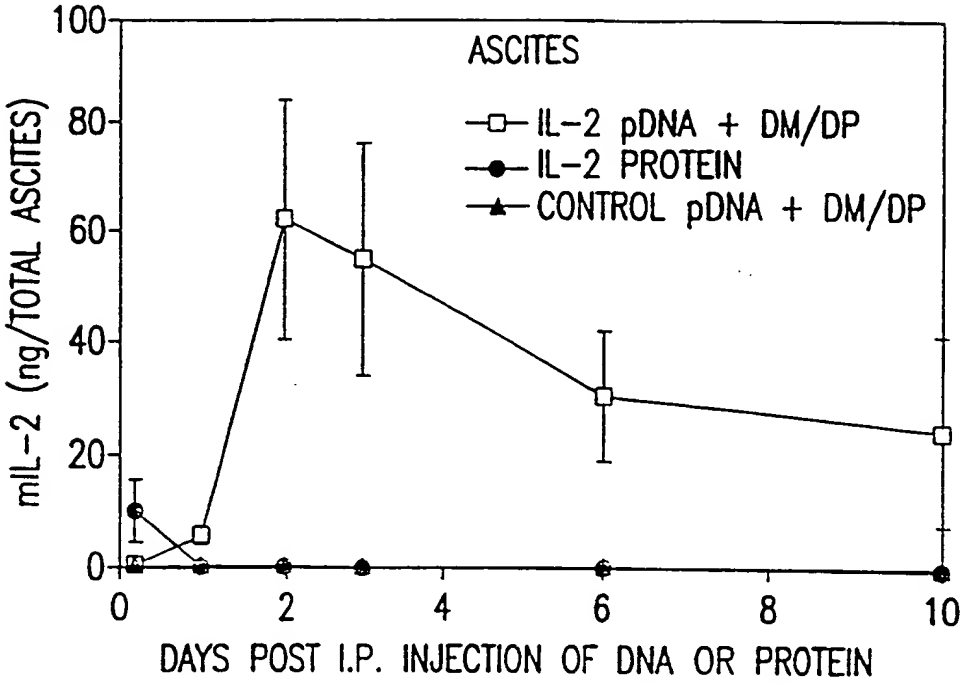

FIG.14A

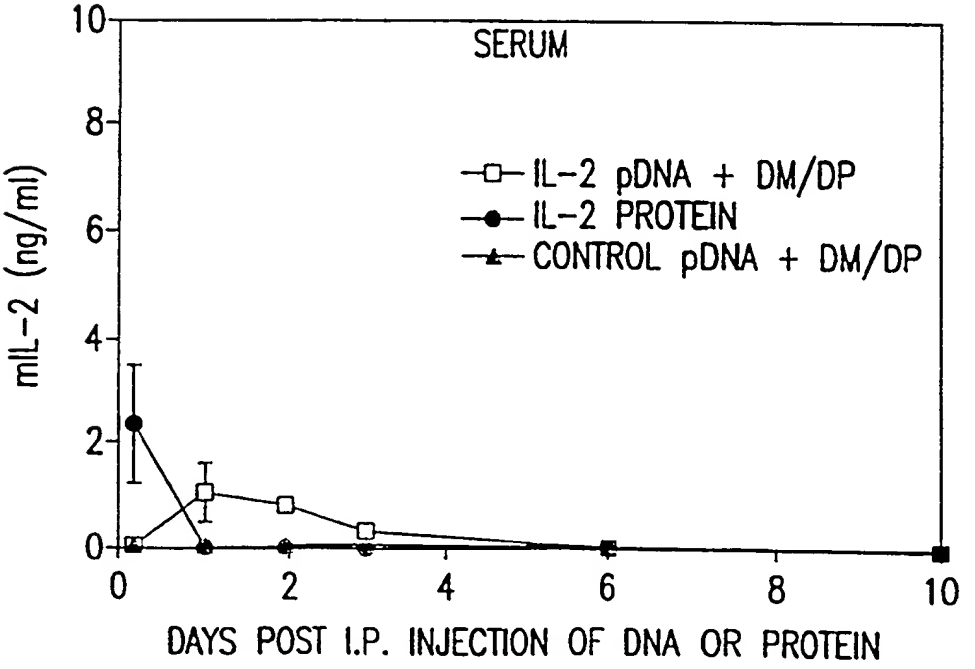

FIG.14B

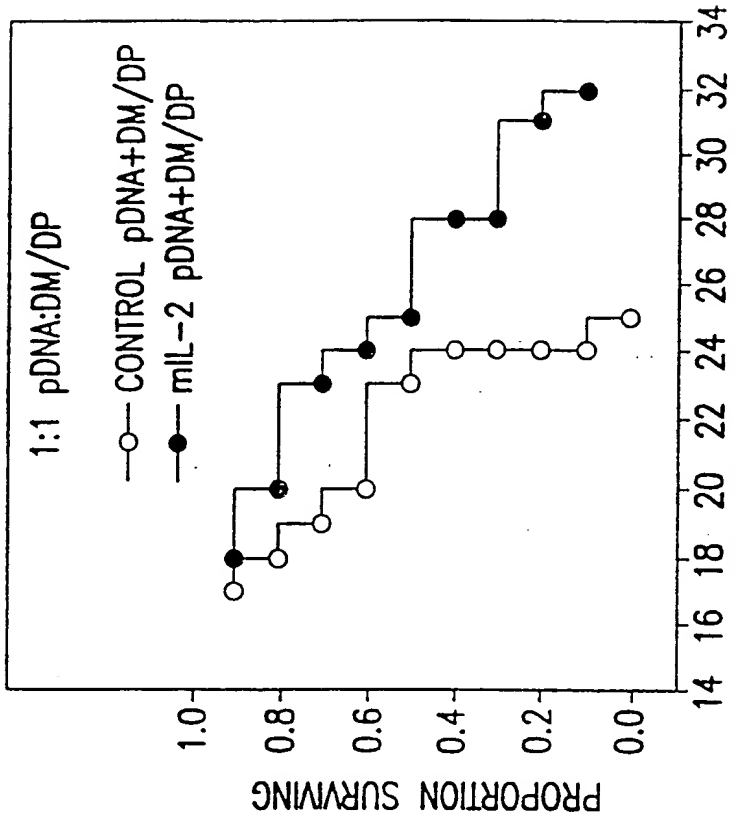

FIG. 15B

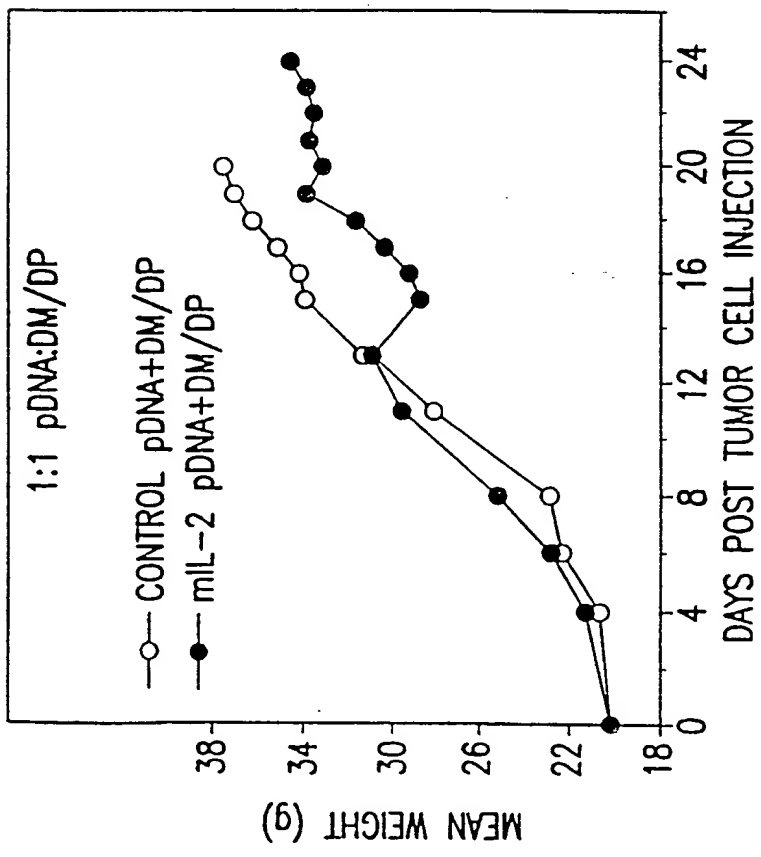

FIG. 15A

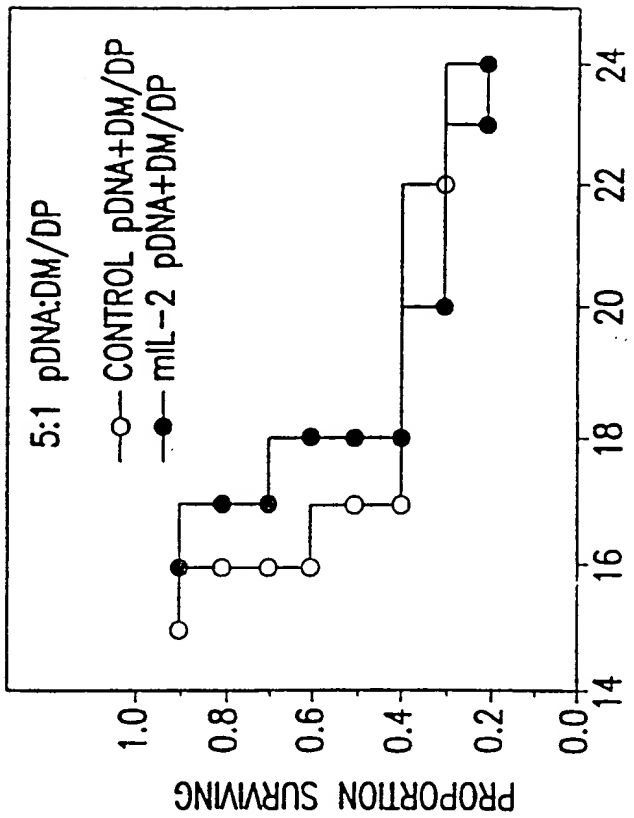

FIG.15D

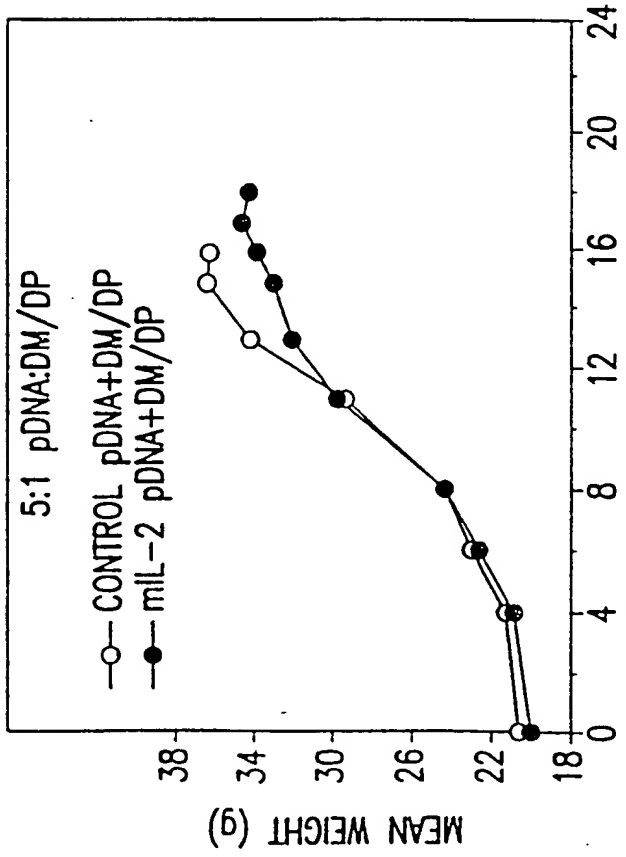

FIG.15C

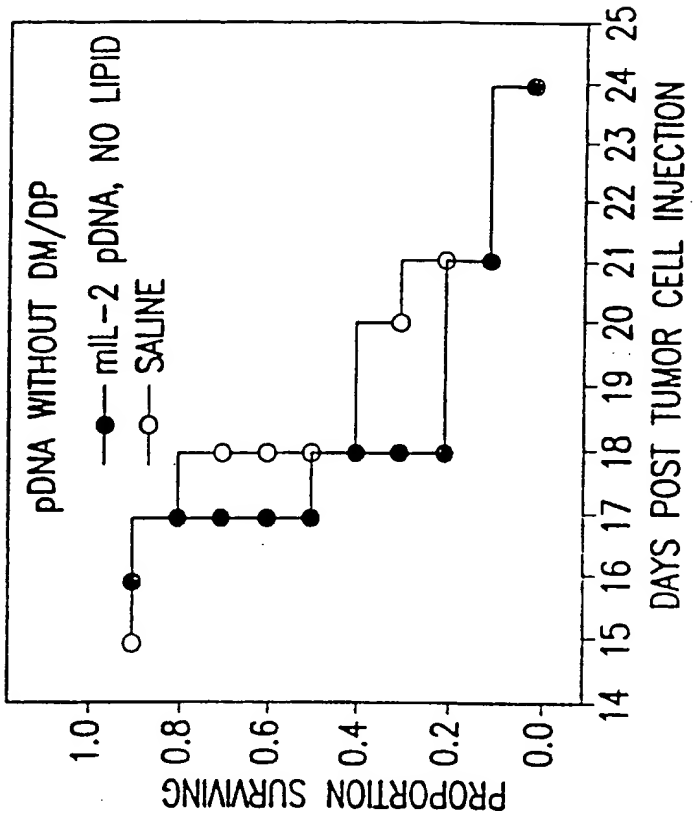

FIG.15F

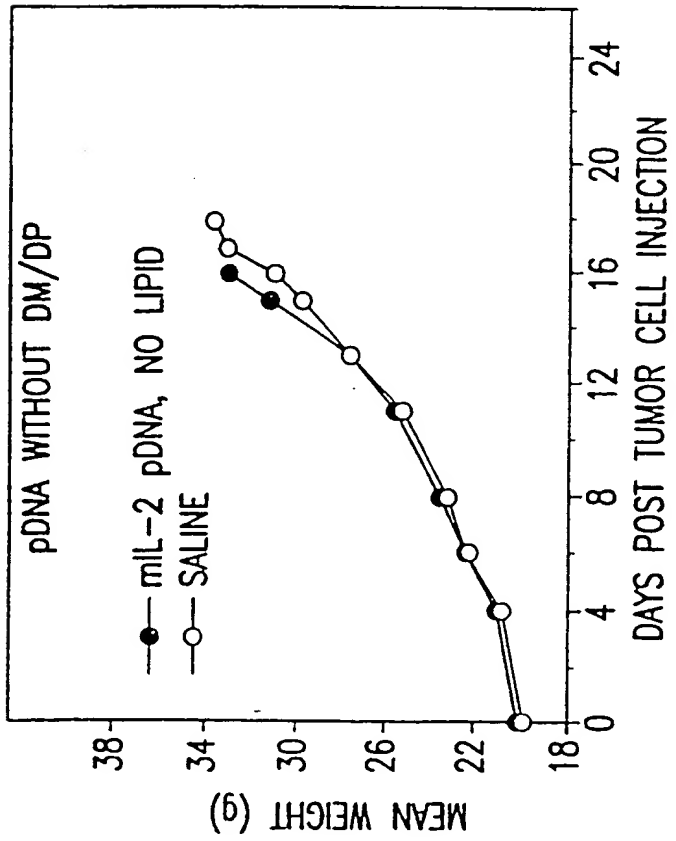

FIG.15E

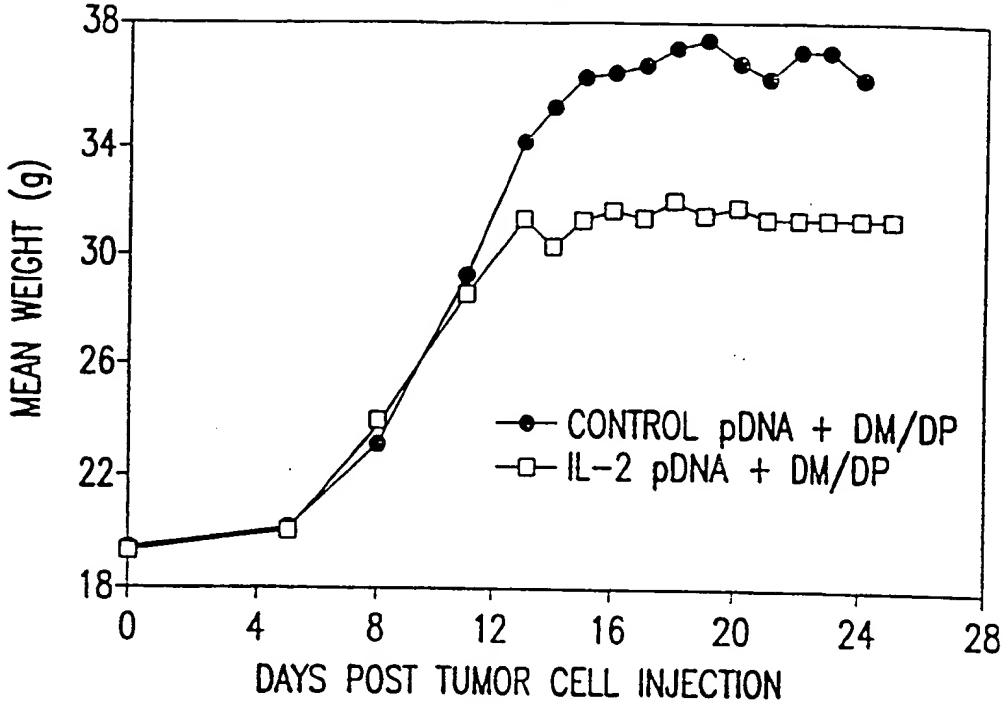

FIG.16A

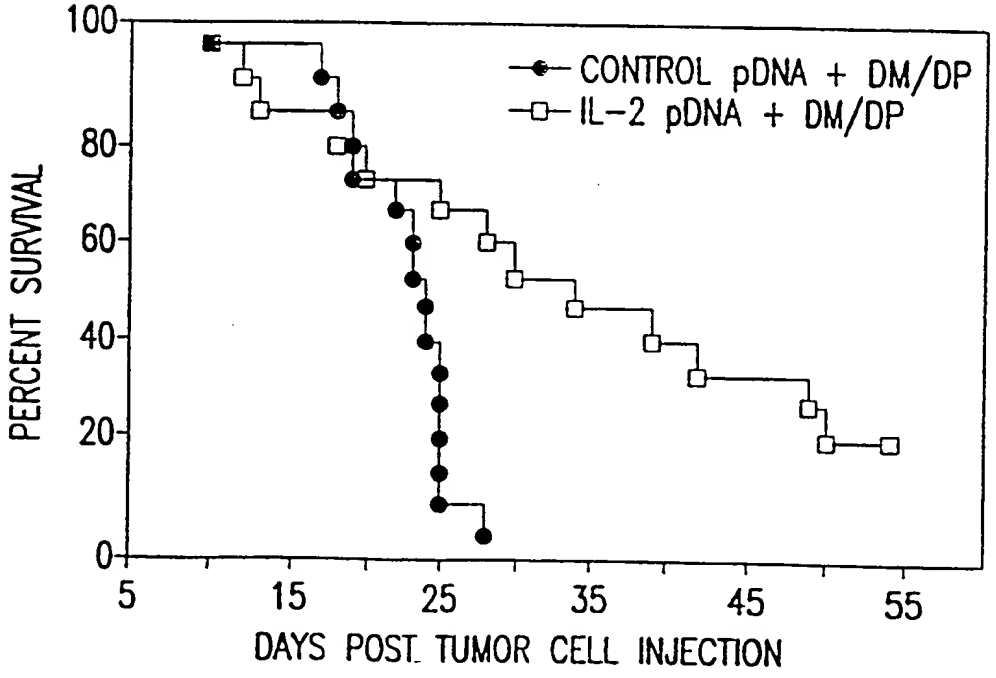

FIG.16B

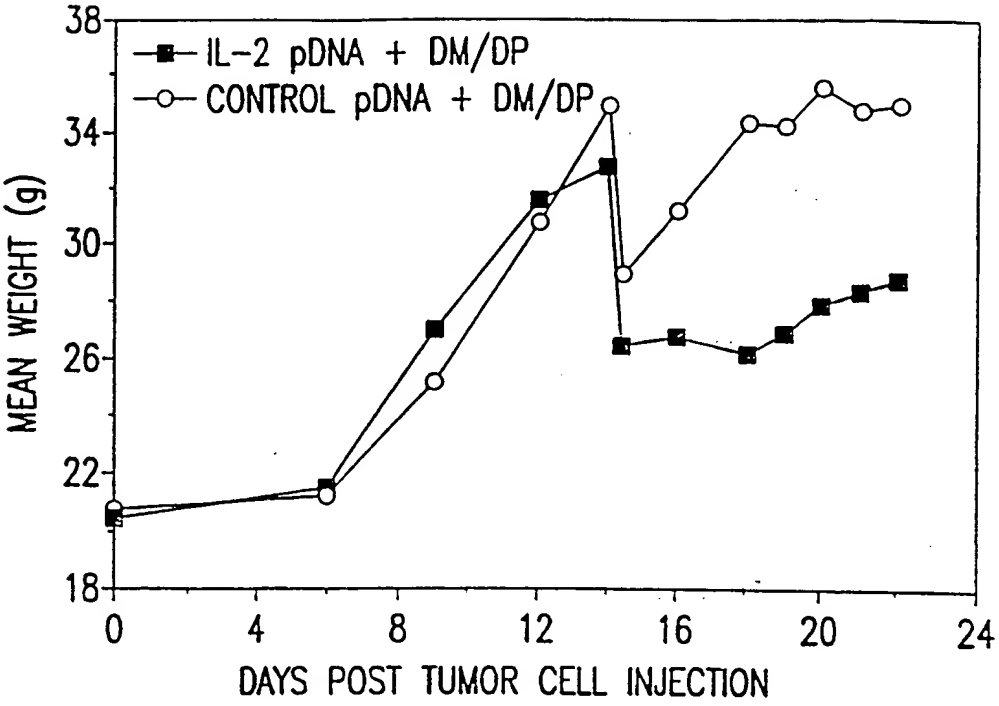

FIG.17A

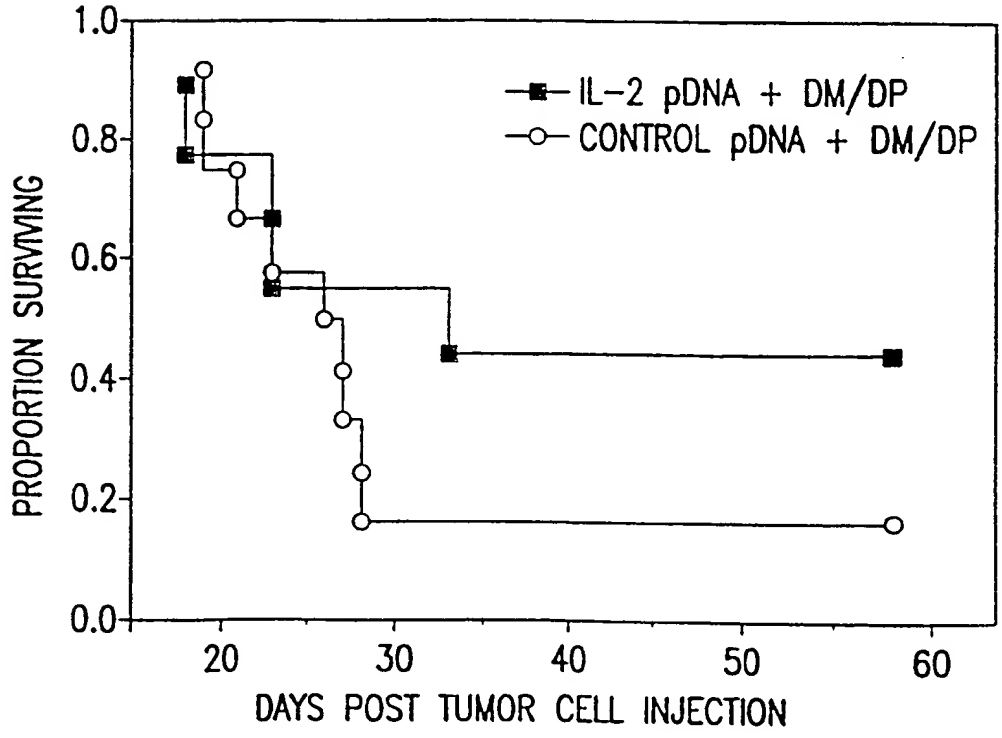

FIG.17B

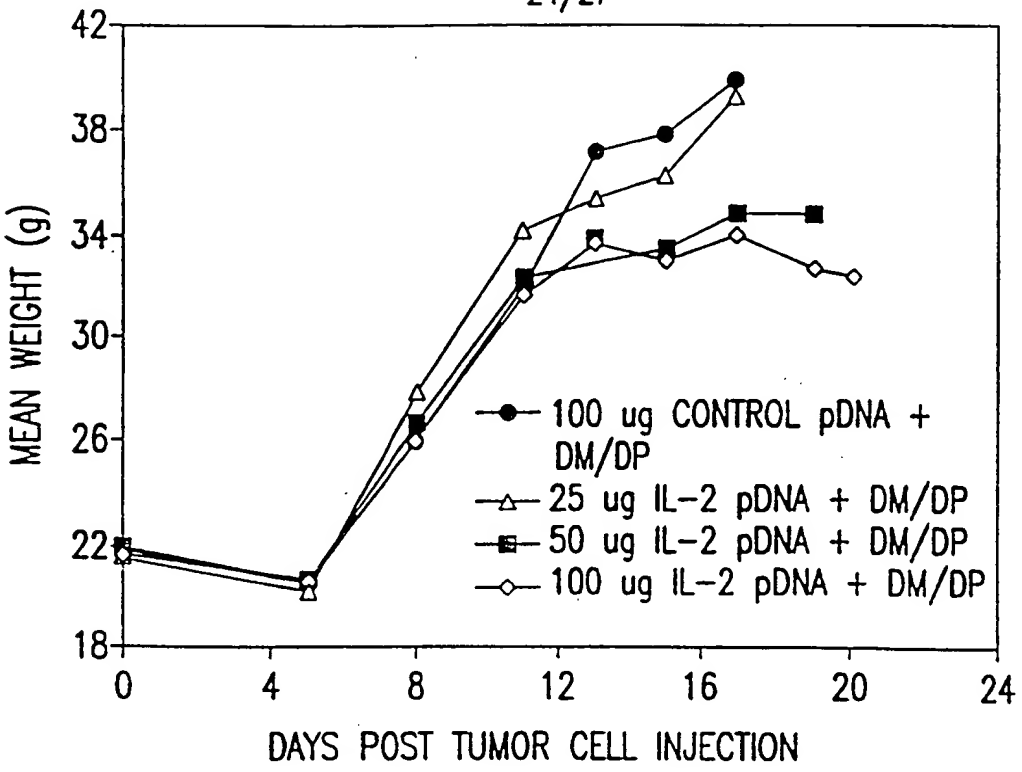

FIG.18A

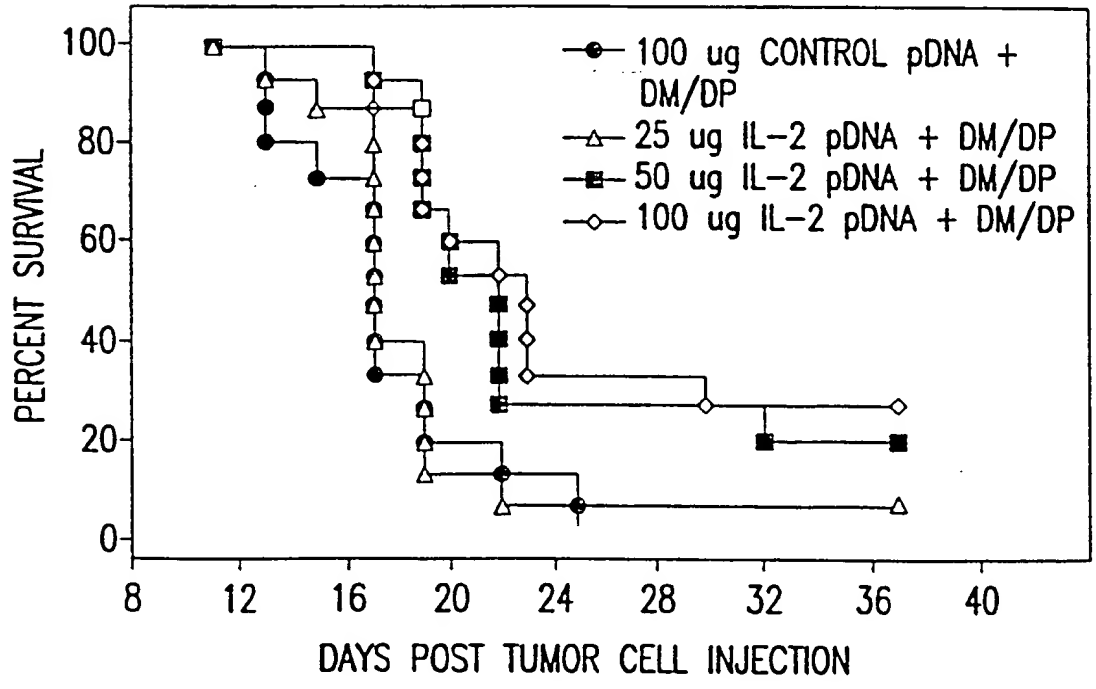

FIG.18B

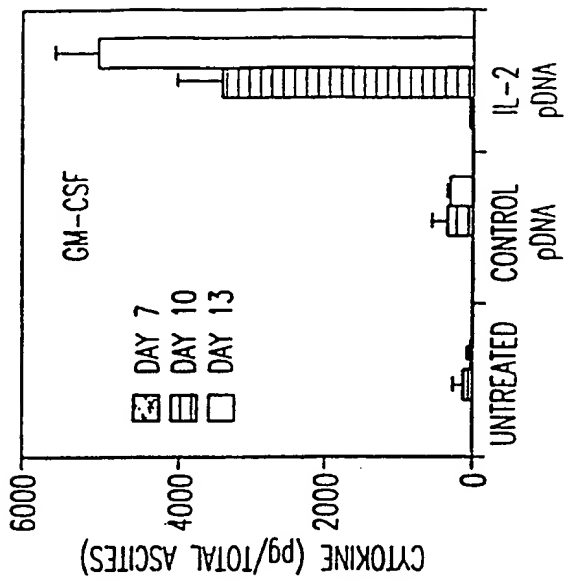

FIG.19C

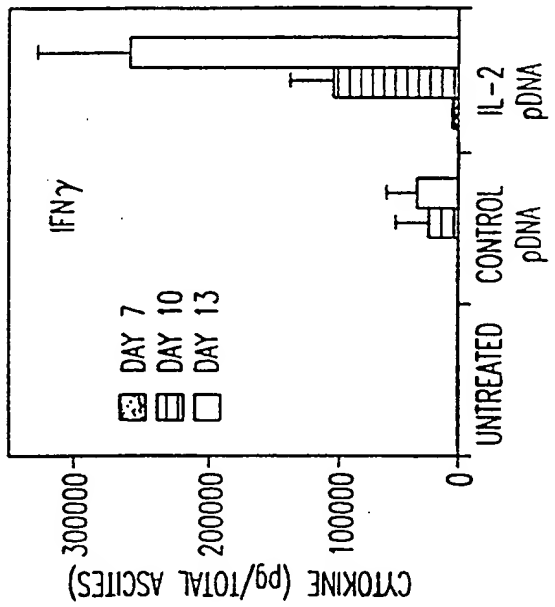

FIG.19B

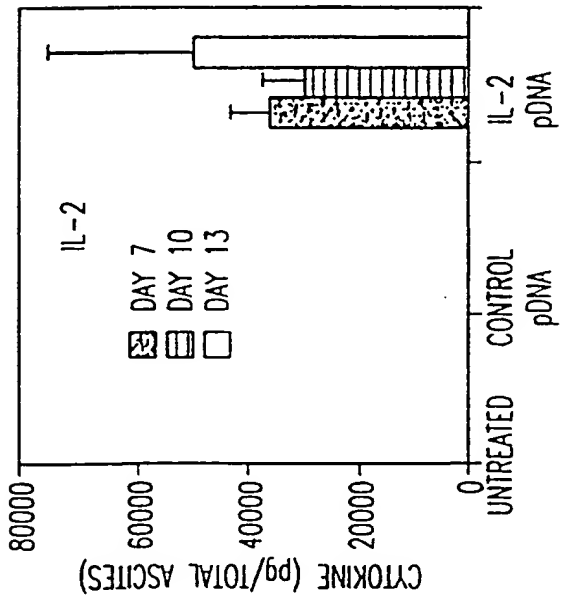

FIG.19A

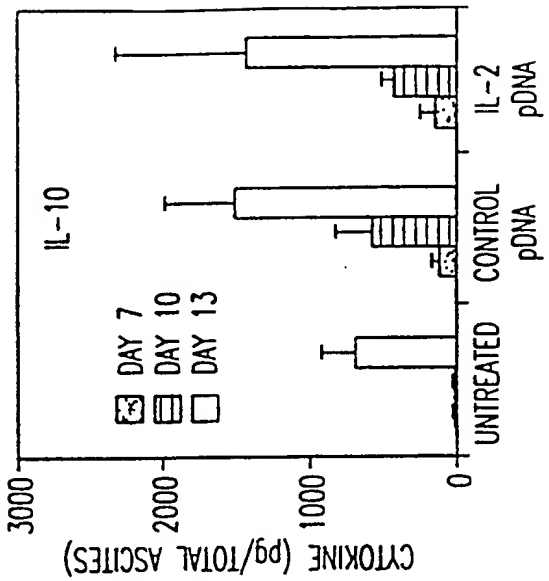

FIG.19F

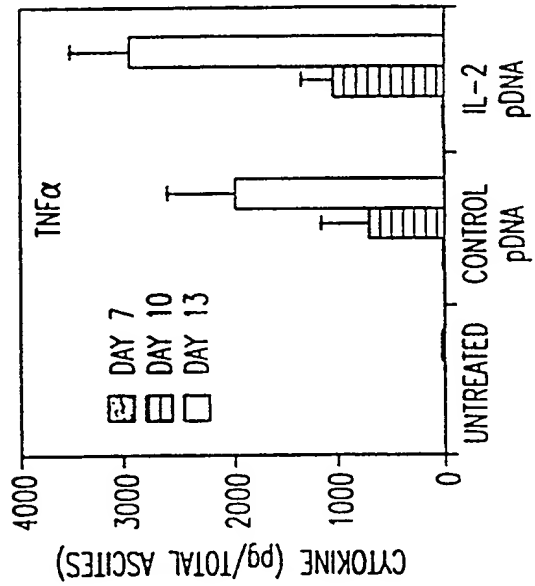

FIG.19E

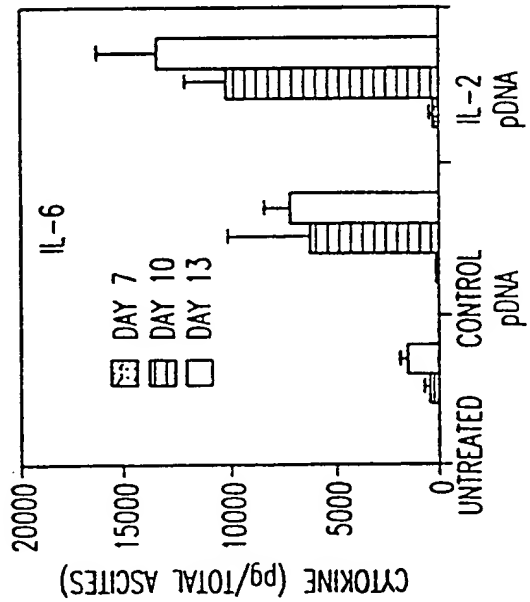

FIG.19D

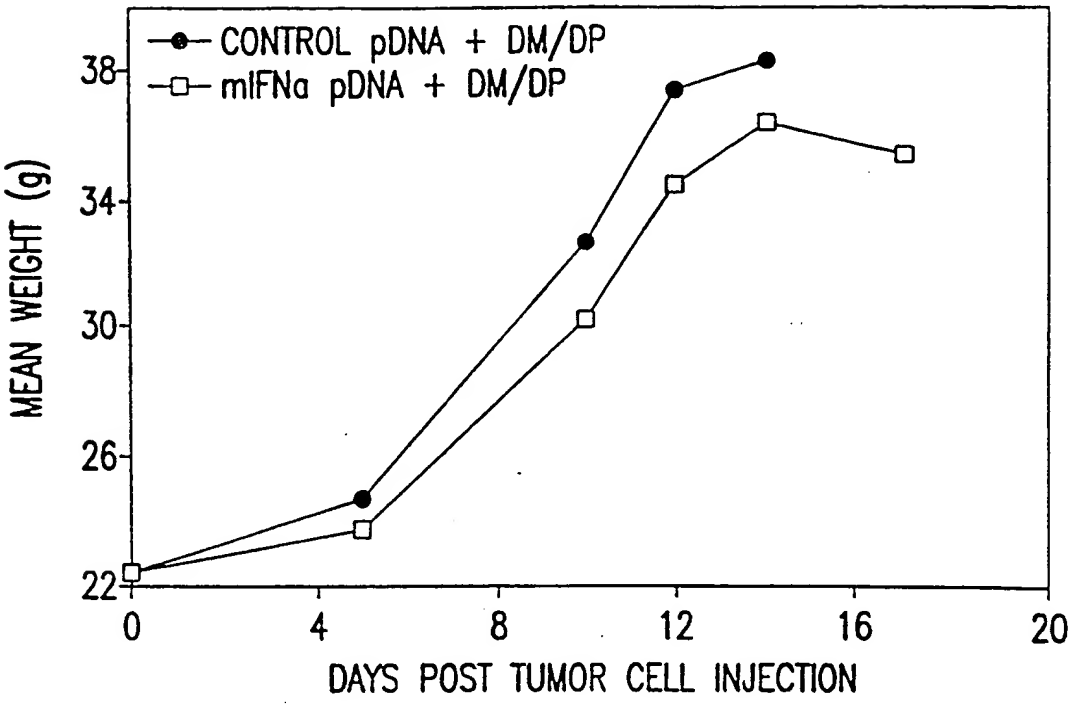

FIG.20A

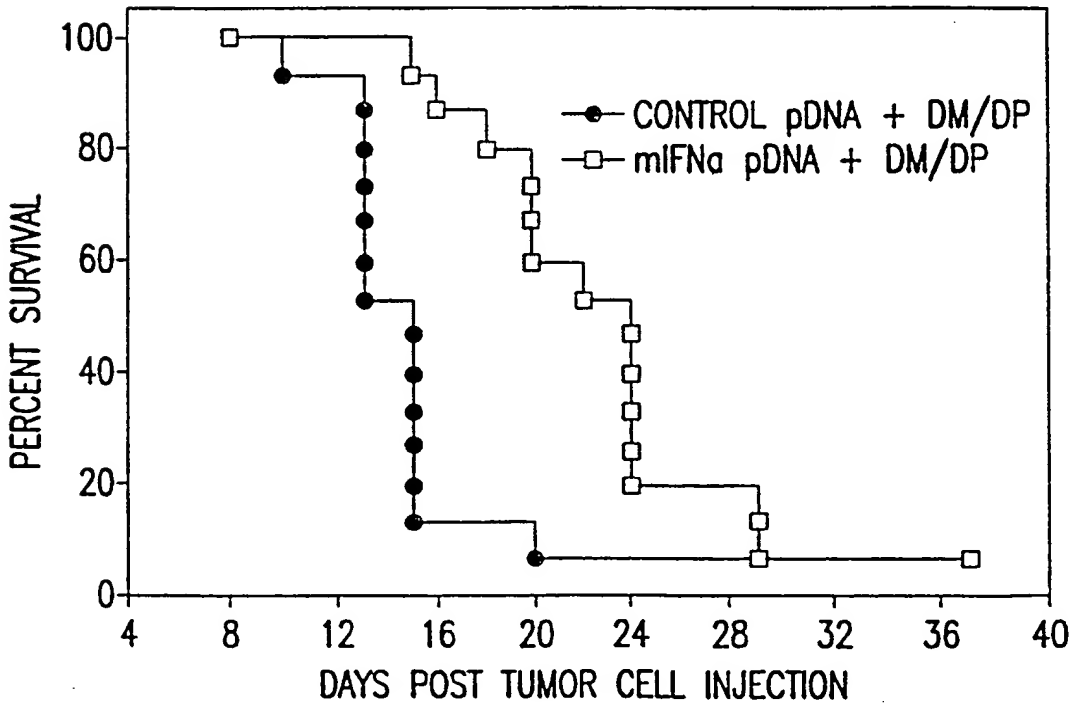

FIG.20B